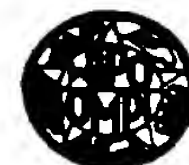


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(54) Title: ANTAGONISTS OF INTERLEUKIN-15

(57) Abstract

Disclosed herein are mutant IL-15 polypeptides and methods for using these polypeptides to modulate the immune response in a patient.

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Summary of the Invention

Mutants of the cytokine IL-15 that bind the IL-15 receptor complex with an affinity similar to wild-type IL-15, but fail to activate signal transduction, have  
5 been produced. These mutant polypeptides compete effectively with wild-type IL-15 and can block one or more of the events that normally occur in response to IL-15 signalling, such as cellular proliferation. By modulating the events mediated by the IL-15 receptor  
10 complex, mutant IL-15 polypeptides can modulate the immune response, and thus are therapeutically useful.

Mutant IL-15 polypeptides can have several characteristics that are advantageous in the context of treatment regimes. First, they are less likely to be  
15 immunogenic because they can differ from wild type IL-15 by only a few substituted residues. Second, IL-15 mutants bind IL-15R $\alpha$  with the same high affinity as wild type IL-15 and thus, can compete effectively for the receptor. Third, IL-15 mutants can be easily modified to  
20 remain active in the circulation for a prolonged period of time, or to produce a lytic response in cells to which they bind. In addition, the IL-15 receptor alpha (IL-15R $\alpha$ ) polypeptide is expressed upon activated or malignant immune cells, but not on resting immune cells.  
25 Thus, the mutant polypeptide of the invention can be used to specifically target activated or malignant immune cells.

Other features and advantages of the invention will be apparent from the following detailed description,  
30 and from the claims. Although materials and methods similar or equivalent to those described herein can be used in the practice or testing of the invention, the preferred materials and methods are described below.

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ANTAGONISTS OF INTERLEUKIN-15Related Application(s)

5        This application claims benefit from a provisional application that was filed on April 26, 1996, and assigned U.S. Serial No. 60/016,634.

Field of the Invention

10        The field of the invention is cytokine-mediated therapeutics.

Background of the Invention

      An effective immune response begins when an antigen or mitogen triggers the activation of T cells. In the process of T cell activation, numerous cellular  
15        changes occur, which include the expression of cytokines and cytokine receptors. One of the cytokines involved in the immune response is interleukin-15 (IL-15). IL-15 is a T cell growth factor that stimulates the proliferation and differentiation of B cells, T cells, natural killer  
20        (NK) cells, and lymphocyte-activated killer (LAK) cells *in vitro*. *In vivo*, the proliferation of these cell types enhances the immune response.

      IL-15 binds to a heterotrimeric receptor that consists of the IL-2R $\beta$  subunit, the IL-2R $\gamma$  subunit, and a  
25        unique IL-15R $\alpha$  subunit.

      Patients benefit from suppression of the immune response in a number of circumstances, for example, in the event of organ transplantation or autoimmune disease. In other circumstances, for example when select immune  
30        cells have become malignant or autoaggressive, it is beneficial to actively destroy them.

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incorporated radioactivity was determined by scintillation counting.

Figs. 4A-4C are a series of plots depicting the expression of IL-15R $\alpha$  subunits on human PBMCs by flow cytometry analysis of stained cells. Freshly isolated or PHA pre-activated PBMCs were washed and incubated with medium alone (thin line) or FLAG-HMK-IL-15 (thick line) followed by anti-FLAG biotinylated Ab and Streptavidin-RED670 (Fig. 4A). The data presented in Fig. 4B were obtained from washed PBMCs that were preincubated with media alone (left-hand side) or media containing 2  $\mu$ g of human recombinant IL-15 (right-hand side) for 20 minutes at 4°C before the addition of FLAG-HMK-IL-15. For simplicity, graphs represent specific binding of FLAG-HMK-IL-15; non-specific binding was subtracted. The data presented in Fig. 4C were obtained from PBMCs that were preincubated with phycoerythrin conjugated anti-CD4 or anti-CD8 for 30 minutes before the addition of FLAG-HMK-IL-15.

Figs. 5A-5C are a series of plots generated by fluorescence activated cell sorting (FACS) analysis of PHA-activated PBMCs stained with FLAG-HMK-IL-15 proteins and anti-CD25 $\beta$  (IL-2R $\alpha$  chain) monoclonal antibodies. Fig. 5A demonstrates the existence of IL-2R $\alpha$ <sup>+</sup> cells that do not bind FLAG-HMK-IL-15. Fig. 5B demonstrates that almost all PBMCs stimulated with PHA for only one day express either IL-15R $\alpha$  or IL-2R $\beta$  subunits, but not both. Fig. 5C demonstrates that a larger population of IL-15R $\alpha$ <sup>+</sup> and IL-2R $\beta$ <sup>+</sup> cells (double positive) are present 3 days following PHA stimulation.

Figs. 6A-6D are a series of plots generated by fluorescence activated cell sorting (FACS). To determine the effect of immunosuppressant drugs on the mitogen-induced expression of IL-15R $\alpha$ , PBMCs were preincubated with cyclosporin (CsA; Fig. 6B), rapamycin (RAPA; Fig.

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Brief Description of the Drawings

Fig. 1 is a photograph of a Western blot depicting the bacterially-produced, affinity-purified FLAG-HMK-IL-15 fusion protein. Proteins were separated by electrophoresis through a 20% SDS-polyacrylamide gel, visualized by staining with Coomassie blue (lane a) and transferred to PVDF membranes. The membranes were exposed to antibodies against the FLAG peptide (lane b) and human IL-15 (lane c). In lane (d), eluates from an anti-FLAG affinity column were incubated with heart muscle kinase and [ $^{32}$ P]- $\gamma$ -ATP for 30 minutes before electrophoresis.

Fig. 2 is an autoradiograph depicting proteins that were extracted from PHA-stimulated PBMCs that were incubated with [ $^{32}$ P]-FLAG-HMK-IL-15, cross-linked with DSS, and separated by SDS-PAGE under reducing conditions. A 75-80 kDa band is apparent (lane 1). To control for nonspecific crosslinking, replicates were incubated with [ $^{32}$ P]-FLAG-HMK-IL-15 in the presence of a molar excess of IL-15 (lane 2).

Fig. 3A is a bar graph depicting the mitogenic response of PHA-stimulated PBMCs that were stimulated with either buffer alone ("none"), FLAG peptide ( $10^{-4}$  M), IL-2 ( $10^{-7}$  M) or FLAG-HMK-IL-15 ( $10^{-9}$  M). The cultured cells were "pulsed" with [ $^3$ H]-TdR, and the amount of incorporated radioactivity was determined by scintillation counting.

Fig. 3B is a bar graph depicting the mitogenic response of BAF-B03 cells transfected with pRcCMV (control; left-hand panel) or with pRcCMV-IL-2R $\beta$  (encoding the wild-type human IL-2R $\beta$  subunit; right-hand panel) and incubated with medium alone ("none"), media containing IL-3, IL-2 (50 U/ml), or FLAG-HMK-IL-15 (10 ng/ml), and pulsed with [ $^3$ H]-TdR (b). The cultured cells were "pulsed" with [ $^3$ H]-TdR, and the amount of

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exogenously added cytokines. Error bars indicate the mean  $\pm$  standard deviation.

Fig. 11 is a graph depicting the effect of cyclosporin-A on the proliferation of PHA-stimulated 5 PBMCs. The cells were incubated with increasing concentrations of cyclosporine-A for 15 minutes at 37°C, followed by stimulation with IL-2 or IL-15 for 38 hours, and a standard proliferation assay was performed. In the control experiment, cells were cultured in media lacking 10 exogenously added cytokines. Error bars indicate the mean  $\pm$  standard deviation.

Fig. 12 is a pair of graphs depicting the proliferative response of BAF-BO3 cells that express the wild-type IL-2R $\beta$  subunit (WT,  $\Delta$ --- $\Delta$ ), the mutant IL-2R $\beta$  15 subunit that lacks serine-rich region (S $\Delta$ ,  $\blacksquare$ --- $\blacksquare$ ), or a control vector (V,  $\bullet$ --- $\bullet$ ). The cells were incubated with IL-2 (upper graph) or IL-15 (lower graph) for 38 hours and a standard proliferation assay was performed. Error bars indicate the mean  $\pm$  standard deviation.

Fig. 13 is a representation of the wild-type IL-15 20 nucleic acid and predicted amino acid sequence.

Fig. 14 is a representation of the mutant IL-15 nucleic acid and predicted amino acid sequence. The wild-type codon encoding glutamine at position 149, CAG, 25 and the wild-type codon encoding glutamine at position 156, CAA, have both been changed to GAC, which encodes aspartate. (These positions (149 and 156) correspond to positions 101 and 108, respectively, in the mature IL-15 polypeptide, which lacks a 48-amino acid signal 30 sequence).

Fig. 15 is a bar graph depicting the proliferative response of BAF-BO3 cells cultured in the presence of IL-15 related polypeptides. Abbreviations: WT=BAF-BO3 cells transfected with pRcCMV-IL-2R $\beta$  (encoding the human 35 IL-2R $\beta$  subunit); V=BAF-BO3 cells transfected with pRcCMV-



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6C), or dexamethasone (Dex; Fig. 6D) for 20 minutes before the addition of PHA and then cultured for 3 days. The control, stimulation with PHA only, is shown in Fig. 6A. The level of IL-15R $\alpha$  expression was detected using  
5 FLAG-HMK-IL-15, anti-FLAG biotinylated Ab, streptavidin-RED670, and FACS analysis.

Fig. 7 is a bar graph depicting the proliferative response of human PBMCs that were pre-treated with immunosuppressive drugs and PHA and then treated with  
10 PHA, IL-2 or IL-15. Cells were harvested after a 4 hour pulse of [<sup>3</sup>H]-TdR and cell-incorporated radioactivity was measured in a scintillation counter.

Fig. 8A is a diagram of the protocol used to induce IL-2 and IL-15 responsive PBMCs.

15 Fig. 8B is a graph depicting the proliferative response of PBMCs that were pre-stimulated with PHA for 3 days and then cultured in the presence of IL-2 ( $\Delta$ ;  $\square$ ) or IL-15 ( $\blacktriangle$ ;  $\blacksquare$ ) for 2 days, and then tested for secondary response to IL-2 or IL-15 according to the protocol  
20 illustrated in Fig. 8A. Arrow indicates that the cytokine was present during secondary stimulation. Error bars indicate the mean  $\pm$  standard deviation.

Fig. 9 is a line graph. PHA-stimulated PBMCs were incubated with genistein at various doses for 15 minutes  
25 at 37°C followed by stimulation with IL-2 or IL-15 for 38 hours in a standard proliferation assay. Control cells were not treated with genistein. Error bars indicate the mean  $\pm$  standard deviation.

Fig. 10 is a graph depicting the effect of  
30 rapamycin on the proliferation of PHA-stimulated PBMCs. Cells were incubated with increasing concentrations of rapamycin for 15 minutes at 37°C, followed by stimulation with IL-2 (o---o) or IL-15 ( $\square$ --- $\square$ ) for 38 hours, and a standard proliferation assay was performed. In the  
35 control experiment, cells were cultured in media lacking

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(tracing alone) or with medium containing the FLAG-HMK-IL-15 polypeptide (tracing filled in). The cells were then incubated with the biotinylated anti-FLAG antibody and stained with RED670-conjugated streptavidin. The  
5 stained cells were analyzed by flow cytometry.

#### Detailed Description

Mutant IL-15 polypeptides can suppress IL-15 dependent immune responses by selectively inhibiting the activity of cells that bind wild-type IL-15. The mutant  
10 can also be used to kill the cells to which it binds.

Accordingly, the invention features a substantially pure polypeptide that is a mutant of wild-type IL-15, nucleic acid molecules that encode this polypeptide, and cells that express the mutant  
15 polypeptide. Methods of treatment, wherein a mutant IL-15 polypeptide is administered to suppress the immune response (for example, in the event of an autoimmune disease) or to kill IL-15-binding cells (for example, in the event of unwanted cellular proliferation of  
20 IL-15-binding cells) are also within the scope of the invention.

#### Mutant IL-15 Polypeptides

The invention features a substantially pure polypeptide that is a mutant of wild-type IL-15, and  
25 which can function as an antagonist of wild-type IL-15. The "wild-type IL-15 polypeptide" referred to herein is a polypeptide that is identical to a naturally-occurring IL-15 (interleukin-15) polypeptide. IL-15 has been characterized as a T cell growth factor that stimulates  
30 the proliferation and differentiation of B cells, T cells, natural killers (NK) cells, and lymphocyte-activated killers (LAK) cells in vitro. In contrast, a "mutant IL-15 polypeptide" is a polypeptide that has at



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0 (plasmid without insert); none=incubation of cells in medium without added interleukin; DM=incubation of cells in medium containing the IL-15 double mutant (FLAG-HMK-IL-15-Q149D-Q156D); 149=incubation of cells in medium  
5 containing the IL-15 single mutant (FLAG-HMK-IL-15-Q149D).

Fig. 16 is a bar graph depicting the proliferative response of PHA-stimulated human PBMCs cultured in the presence of IL-15 related proteins. Abbreviations:  
10 WT=BAF-BO3 cells transfected with pRcCMV-IL-2R $\beta$  (encoding the human IL-2R $\beta$  subunit); V=BAF-BO3 cells transfected with pRcCMV-0 (plasmid without insert); none=incubation of cells in medium without added interleukin;  
DM=incubation of cells in medium containing the IL-15  
15 double mutant (FLAG-HMK-IL-15-Q149D-Q156D);  
149=incubation of cells in medium containing the IL-15 single mutant (FLAG-HMK-IL-15-Q149D); 156=incubation of cells in medium containing the IL-15 single mutant (FLAG-HMK-IL-15-Q156D).

20 Fig. 17 is a series of plots demonstrating the ability of the FLAG-HMK-IL-15 double mutant to bind PHA-activated human PBMCs. PHA-activated PBMCs were washed and incubated with medium alone (a) or with FLAG-HMK-IL-15 double mutants (b) followed by anti-FLAG  
25 biotinylated Ab and streptavidin-RED670. The stained cells were analyzed by flow cytometry. In (c), (a) and (b) are overlapped (control=thin line, FLAG-HMK-IL-15 double mutant=thick line).

Fig. 18 is a series of plots demonstrating the  
30 ability of the wild-type FLAG-HMK-IL-15 polypeptide to bind leukemia cells. The cells treated were from the leukemic cell lines MOLT-14, YT, HuT-102, and from cell lines currently being established at Beth Israel Hospital (Boston, MA), and designated 2A and 2B. The cultured  
35 cells were washed and incubated with either medium alone

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reference polypeptide over its entire length. Of course, many other polypeptides will meet the same criteria.

Sequence identity is typically measured using sequence analysis software such as the Sequence Analysis Software Package of the Genetics Computer Group at the University of Wisconsin Biotechnology Center (1710 University Avenue, Madison, WI 53705), with the default parameters thereof.

The mutant polypeptide can be at least 65%, preferably at least 80%, more preferably at least 90%, and most preferably at least 95% (e.g., 99%) identical to wild-type IL-15. The mutation can consist of a change in the number or content of amino acid residues. For example, the mutant IL-15 can have a greater or a lesser number of amino acid residues than wild-type IL-15. Alternatively, or in addition, the mutant polypeptide can contain a substitution of one or more amino acid residues that are present in the wild-type IL-15. The mutant IL-15 polypeptide can differ from wild-type IL-15 by the addition, deletion, or substitution of a single amino acid residue, for example, a substitution of the residue at position 156. Similarly, the mutant polypeptide can differ from wild-type by a substitution of two amino acid residues, for example, the residues at positions 156 and 149. For example, the mutant IL-15 polypeptide can differ from wild-type IL-15 by the substitution of aspartate for glutamine at residues 156 and 149 (as shown in Figures 14 and 15). Additions, deletions, and substitutions of more than two residues, and substitutions at other positions are likely to produce a similarly useful (i.e., therapeutically effective) mutant IL-15 polypeptide.

The substituted amino acid residue(s) can be, but are not necessarily, conservative substitutions, which typically include substitutions within the following

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least one mutation relative to wild-type IL-15 and that inhibits any in vivo or in vitro activity that is characteristic of wild-type IL-15.

A mutant IL-15 polypeptide that can be used according to the present invention will generally blocks at least 40%, more preferably at least 70%, and most preferably at least 90% of one or more of the activities of the wild-type IL-15 molecule. The ability of a mutant IL-15 polypeptide to block wild-type IL-15 activity can be assessed by numerous assays, including the BAF-BO3 cell proliferation assay described herein (in which the cells were transfected with a construct encoding IL-2R $\beta$ ).

The mutant polypeptides of the invention can be referred to as exhibiting a particular percent identity to wild-type IL-15. When examining the percent identity between two polypeptides, the length of the sequences compared will generally be at least 16 amino acids, preferably at least 20 amino acids, more preferably at least 25 amino acids, and most preferably at least 35 amino acids.

The term "identity" as used herein in reference to polypeptide or DNA sequences, refers to the subunit sequence identity between two molecules. When a subunit position in both of the molecules is occupied by the same monomeric subunit (i.e., the same amino acid residue or nucleotide), then the molecules are identical at that position. The similarity between two amino acid or two nucleotide sequences is a direct function of the number of identical positions. Thus, a polypeptide that is 50% identical to a reference polypeptide that is 100 amino acids long can be a 50 amino acid polypeptide that is completely identical to a 50 amino acid long portion of the reference polypeptide. It might also be a 100 amino acid long polypeptide that is 50% identical to the

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polypeptide; as described further below, native activity is not necessary or desired in all cases.

The Fc region can be "lytic" or "non-lytic." A non-lytic Fc region typically lacks a high affinity Fc receptor binding site and a C'1q binding site. The high affinity Fc receptor binding site of murine IgG Fc includes the Leu residue at position 235 of IgG Fc. Thus, the Fc receptor binding site can be destroyed by mutating or deleting Leu 235. For example, substitution of Glu for Leu 235 inhibits the ability of the Fc region to bind the high affinity Fc receptor. The murine C'1q binding site can be functionally destroyed by mutating or deleting the Glu 318, Lys 320, and Lys 322 residues of IgG. For example, substitution of Ala residues for Glu 318, Lys 320, and Lys 322 renders IgG1 Fc unable to direct antibody-dependent complement lysis. In contrast, a lytic IgG Fc region has a high affinity Fc receptor binding site and a C'1q binding site. The high affinity Fc receptor binding site includes the Leu residue at position 235 of IgG Fc, and the C'1q binding site includes the Glu 318, Lys 320, and Lys 322 residues of IgG1. Lytic IgG Fc has wild-type residues or conservative amino acid substitutions at these sites. Lytic IgG Fc can target cells for antibody dependent cellular cytotoxicity or complement directed cytolysis (CDC). Appropriate mutations for human IgG are also known (see, e.g., Morrison et al., *The Immunologist* 2:119-124, 1994; and Brekke et al., *The Immunologist* 2:125, 1994).

In other instances, the chimeric polypeptide may include the mutant IL-15 polypeptide and a polypeptide that functions as an antigenic tag, such as a FLAG sequence. FLAG sequences are recognized by biotinylated, highly specific, anti-FLAG antibodies, as described herein (see also Blonar et al., *Science* 256:1014, 1992;

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groups: glycine, alanine; valine, isoleucine, leucine; aspartic acid, glutamic acid; asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine.

5       The mutations described above can be in the carboxy-terminal domain of the cytokine, which is believed to bind the IL-2R $\gamma$  subunit. It is also possible that one or more mutations can be within the IL-2R $\beta$  binding domain.

10       In a related aspect, the invention features a chimeric polypeptide that includes a mutant IL-15 polypeptide and a heterologous polypeptide (i.e., a polypeptide that is not IL-15 or a mutant thereof). The heterologous polypeptide can increase the circulating  
15 half-life of the chimeric polypeptide in vivo. The polypeptide that increases the circulating half-life may be a serum albumin, such as human serum albumin, or the Fc region of the IgG subclass of antibodies that lacks the IgG heavy chain variable region. The Fc region may  
20 include a mutation that inhibits complement fixation and Fc receptor binding, or it may be lytic, i.e., able to bind complement or to lyse cells via another mechanism, such as antibody-dependent complement lysis (ADCC; 08/355,502 filed 12/12/94).

25       The "Fc region" can be a naturally-occurring or synthetic polypeptide that is homologous to the IgG C-terminal domain produced by digestion of IgG with papain. IgG Fc has a molecular weight of approximately 50 kDa. The polypeptides of the invention can include  
30 the entire Fc region, or a smaller portion that retains the ability to extend the circulating half-life of a chimeric polypeptide of which it is a part. In addition, full-length or fragmented Fc regions can be variants of the wild-type molecule. This is, they can contain  
35 mutations that may or may not affect the function of the



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facilitate purification of bacterially expressed protein, or to a hemagglutinin tag to facilitate purification of protein expressed in eukaryotic cells.

The techniques that are required to make mutant IL-15 polypeptides are routine in the art, and can be performed without resort to undue experimentation by artisans of ordinary skill. For example, a mutation that consists of a substitution of one or more of the amino acid residues in IL-15 can be created using the PCR-assisted mutagenesis technique described herein for the creation of the mutant IL-15 polypeptide in which glutamine residues at positions 149 and 156 were changed to aspartic acid residues. Mutations that consists of deletions or additions of amino acid residues to an IL-15 polypeptide can also be made with standard recombinant techniques. In the event of a deletion or addition, the nucleic acid molecule encoding IL-15 is simply digested with an appropriate restriction endonuclease. The resulting fragment can either be expressed directly, or manipulated further, for example, by ligating it to a second fragment. The ligation may be facilitated if the two ends of the nucleic acid molecules contain complementary nucleotides that overlap one another, but blunt-ended fragments can also be ligated.

25 In addition to generating mutant polypeptides via expression of nucleic acid molecules that have been altered by recombinant molecular biological techniques, mutant polypeptides can be chemically synthesized. Chemically synthesized polypeptides are routinely generated by those of skill in the art.



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LeClair et al., Proc. Natl. Acad. Sci. USA 89:8145, 1992).

Chimeric polypeptides can be constructed using no more than conventional molecular biological techniques, which are well within the ability of those of ordinary skill in the art to perform.

As used herein, the terms "protein" and "polypeptide" both refer to any chain of amino acid residues, regardless of length or post-translational modification (e.g., glycosylation or phosphorylation). The proteins or polypeptides of the invention are "substantially pure," meaning that they are at least 60% by weight (dry weight) the polypeptide of interest, e.g., a polypeptide containing the mutant IL-15 amino acid sequence. Preferably, the polypeptide is at least 75%, more preferably at least 90%, and most preferably at least 99%, by weight, the polypeptide of interest.

Purity can be measured by any appropriate standard method, e.g., column chromatography, polyacrylamide gel electrophoresis, or HPLC analysis.

Although the polypeptide can be naturally occurring, it is likely that the polypeptides used in the practice of the instant invention will be synthetic, or produced by expression of a recombinant nucleic acid molecule. Polypeptides that are derived from eukaryotic organisms or synthesized in *E. coli*, or other prokaryotes, and polypeptides that are chemically synthesized will be substantially free from their naturally associated components. In the event the polypeptide is a chimera, it can be encoded by a hybrid nucleic acid molecule (such as those discussed further below) containing one sequence that encodes all or part of the mutant IL-15, and a second sequence that encodes all or part of a second gene. For example, the mutant IL-15 polypeptide may be fused to a hexa-histidine tag to

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techniques. Once the heart is grafted in place and warmed to 37°C with Ringer's lactate solution, normal sinus rhythm will resume. Function of the transplanted heart can be assessed frequently by palpation of ventricular contractions through the abdominal wall. Rejection is defined as the cessation of myocardial contractions, which can be confirmed by under anesthesia. Mutant IL-15 polypeptides would be considered effective in reducing organ rejection if hosts that received injections, for example, of the mutant IL-15 polypeptide experienced longer heart engraftment than the untreated hosts.

#### Skin Grafting

The effectiveness of mutant IL-15 polypeptides can also be assessed following a skin graft. To perform skin grafts on a rodent, a donor animal is anesthetized and the full thickness skin is removed from a part of the tail. The recipient animal is also anesthetized, and a graft bed is prepared by removing a patch of skin from the shaved flank. Generally, the patch is approximately 0.5 x 0.5 cm. The skin from the donor is shaped to fit the graft bed, positioned, covered with gauze, and bandaged. The grafts can be inspected daily beginning on the sixth post-operative day, and are considered rejected when more than half of the transplanted epithelium appears to be non-viable. Mutant IL-15 polypeptides would be considered effective in reducing rejection of the skin grafts if hosts that received injections, for example, of the mutant IL-15 polypeptide tolerated the graft longer than the untreated hosts.

#### Islet Allograft Model

DBA/2J islet cell allografts can be transplanted into rodents, such as 6-8 week-old IL-4<sup>-/-</sup> and IL-4<sup>+/+</sup> mice

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Procedures for Screening Mutant IL-15 Polypeptides

In addition to testing a candidate mutant IL-15 polypeptide in the *in vitro* assays described in the examples below, the following procedures can be employed to determine whether a mutant IL-15 polypeptide is capable of functioning as an antagonist of IL-15 *in vivo*.

Transplantation Paradigms

In order to determine whether a polypeptide is capable of functioning as an immunosuppressant, it can be administered, either directly or by genetic therapy, in the context of well-established transplantation paradigms.

A putative immunosuppressing polypeptide, or a nucleic acid molecule encoding it, could be systemically or locally administered by standard means to any conventional laboratory animal, such as a rat, mouse, rabbit, guinea pig, or dog, before an allogeneic or xenogeneic skin graft, organ transplant, or cell implantation is performed on the animal. Strains of mice such as C57B1-10, B10.BR, and B10.AKM (Jackson Laboratory, Bar Harbor, ME), which have the same genetic background but are mismatched for the H-2 locus, are well suited for assessing various organ grafts.

Heart Transplantation

A method for performing cardiac grafts by anastomosis of the donor heart to the great vessels in the abdomen of the host was first published by Ono et al. (J. Thorac. Cardiovasc. Surg. 57:225, 1969; see also Corry et al., Transplantation 16:343, 1973). According to this surgical procedure, the aorta of a donor heart is anastomosed to the abdominal aorta of the host, and the pulmonary artery of the donor heart is anastomosed to the adjacent vena cava using standard microvascular

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their F<sub>1</sub> hybrids. These animals can be crossed in order to study particular aspects of the rheumatic disease process; progeny of the NZB strain develop severe lupus glomerulonephritis when crossed with NZW mice

5 (Bielschowsky et al., Proc. Univ. Otago Med. Sch. 37:9, 1959; see also *Fundamental Immunology*, Paul, Ed., Raven Press, New York, NY, 1989). Similarly, a shift to lethal nephritis is seen in the progeny of NBZ X SWR matings (Data et al., Nature 263:412, 1976). The histological

10 appearance of renal lesions in SNF<sub>1</sub> mice has been well characterized (Eastcott et al., J. Immunol. 131:2232, 1983; see also *Fundamental Immunology*, supra).

Therefore, the general health of the animal as well as the histological appearance of renal tissue can be used

15 to determine whether the administration of a mutant IL-15 polypeptide can effectively suppress the immune response in an animal model of SLE.

One of the MRL strains of mice that develops SLE, MRL-lpr/lpr, also develops a form of arthritis that

20 resembles rheumatoid arthritis in humans (Theofilopoulos et al., Adv. Immunol. 37:269, 1985). Alternatively, an experimental arthritis can be induced in rodents by

injecting rat type II collagen (2 mg/ml) mixed 1:1 in Freund's complete adjuvant (100  $\mu$ l total) into the base

25 of the tail. Arthritis develops 2-3 weeks after immunization. The ability of nucleic acid molecules encoding mutant IL-15 polypeptides to combat the arthritic condition can be assessed by targeting the nucleic acid molecules to T lymphocytes. One way to

30 target T lymphocytes is the following: spleen cell suspensions are prepared 2-3 days after the onset of arthritis and incubated with collagen (100  $\mu$ g/ml) for 48 hours to induce proliferation of collagen-activated T cells. During this time, the cells are transduced with

35 a vector encoding the polypeptide of interest. As a

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- rendered diabetic by a single intraperitoneal injection of streptozotocin (225 mg/kg; Sigma Chemical Co., St. Louis, MO). As a control, syngeneic IL-4<sup>-/-</sup> islet cell grafts can be transplanted into diabetic IL-4<sup>-/-</sup> mice.
- 5 Islet cell transplantation can be performed by following published protocols (for example, see Gotoh et al., Transplantation 42:387, 1986). Briefly, donor pancreata are perfused *in situ* with type IV collagenase (2 mg/ml; Worthington Biochemical Corp., Freehold, NJ). After a
- 10 40 minute digestion period at 37°C, the islets are isolated on a discontinuous Ficoll gradient. Subsequently, 300-400 islets are transplanted under the renal capsule of each recipient. Allograft function can be followed by serial blood glucose measurements (Accu-
- 15 Check III<sup>™</sup>; Boehringer, Mannheim, Germany). Primary graft function is defined as a blood glucose level under 11.1 mmol/l on day 3 post-transplantation, and graft rejection is defined as a rise in blood glucose exceeding 16.5 mmol/l (on each of at least 2 successive days)
- 20 following a period of primary graft function.

#### Models of Autoimmune Disease

- Models of autoimmune disease provide another means to assess polypeptides *in vivo*. These models are well known to skilled artisans and can be used to determine
- 25 whether a given mutant IL-15 polypeptide is an immunosuppressant that would be therapeutically useful in treating a specific autoimmune disease when delivered either directly or via genetic therapy.

- Autoimmune diseases that have been modeled in
- 30 animals include rheumatic diseases, such as rheumatoid arthritis and systemic lupus erythematosus (SLE), type I diabetes, and autoimmune diseases of the thyroid, gut, and central nervous system. For example, animal models of SLE include MRL mice, BXSB mice, and NZB mice and



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induced by a single injection of brain or spinal cord tissue with adjuvant in many different laboratory animals, including rodents and primates. This model, referred to as experimental allergic encephalomyelitis (EAE) is T cell mediated. Similarly, experimentally induced myasthenia gravis can be produced by a single injection of acetylcholine receptor with adjuvants (Lennon et al., Ann. N.Y. Acad. Sci. 274:283, 1976).

Autoimmune diseases of the gut can be modeled in IL-2 or IL-10 "knock out" mice, or in mice that receive enemas containing bovine serum albumin.

#### Nucleic Acid Molecules Encoding Mutant IL-15

The mutant IL-15 polypeptide, either alone or as a part of a chimeric polypeptide, such as those described above, can be obtained by expression of a nucleic acid molecule. Thus, nucleic acid molecules encoding polypeptides containing a mutant IL-15 are considered within the scope of the invention. Just as the mutant IL-15 polypeptides can be described in terms of their identity with wild-type IL-15 polypeptides, the nucleic acid molecules encoding them will necessarily have a certain identity with those that encode wild-type IL-15. For example, the nucleic acid molecule encoding a mutant IL-15 polypeptide can be at least 65%, preferably at least 75%, more preferably at least 85%, and most preferably at least 95% (e.g., 99%) identical to the nucleic acid encoding wild-type IL-15. For nucleic acids, the length of the sequences compared will generally be at least 50 nucleotides, preferably at least 60 nucleotides, more preferably at least 75 nucleotides, and most preferably 110 nucleotides.

The nucleic acid molecules of the invention can contain naturally occurring sequences, or sequences that differ from those that occur naturally, but, due to the



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control, parallel cultures are grown but not transduced or, transduced with the "empty" vector. The cells are then injected intraperitoneally ( $5 \times 10^7$  cells/animal). The effectiveness of the treatment is assessed by following the disease symptoms during the subsequent 2 weeks, as described by Chernajovsky et al. (*Gene Therapy* 2:731-735, 1995). Lesser symptoms, compared to control, indicate that the peptide of interest, and the nucleic acid molecule encoding it, function as an immunosuppressant potentially useful in the treatment of autoimmune disease.

The ability of a mutant IL-15 polypeptide to suppress the immune response in the case of Type I diabetes can be tested in the BB rat strain, which was developed from a commercial colony of Wistar rats at the Bio-Breeding Laboratories in Ottawa. These rats spontaneously develop autoantibodies against islet cells and insulin, just as occurs with human Type I diabetes. Alternatively, NOD (non-obese diabetic) mice can be used as a model system.

Autoimmune diseases of the thyroid have been modeled in the chicken. Obese strain (OS) chickens consistently develop spontaneous autoimmune thyroiditis resembling Hashimoto's disease (Cole et al., *Science* 160:1357, 1968). Approximately 15% of these birds produce autoantibodies to parietal cells of the stomach, just as in the human counterpart of autoimmune thyroiditis. The manifestations of the disease in OS chickens, which could be monitored in the course of mutant IL-15 polypeptide treatment, include body size, fat deposit, serum lipids, cold sensitivity, and infertility.

Models of autoimmune disease in the central nervous system (CNS) can be experimentally induced. An inflammation of the CNS, which leads to paralysis, can be

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As described above, the mutant IL-15 polypeptide of the invention may exist as a part of a chimeric polypeptide. In addition to, or in place of, the heterologous polypeptides described above, a nucleic acid molecule of the invention can contain sequences encoding a "marker" or "reporter." Examples of marker or reporter genes include  $\beta$ -lactamase, chloramphenicol acetyltransferase (CAT), adenosine deaminase (ADA), aminoglycoside phosphotransferase (neo<sup>r</sup>, G418<sup>r</sup>), dihydrofolate reductase (DHFR), hygromycin-B-phosphotransferase (HPH), thymidine kinase (TK), lacZ (encoding  $\beta$ -galactosidase), and xanthine guanine phosphoribosyltransferase (XGPRT). As with many of the standard procedures associated with the practice of the invention, skilled artisans will be aware of additional useful reagents, for example, of additional sequences that can serve the function of a marker or reporter.

The nucleic acid molecules of the invention can be obtained by introducing a mutation into IL-15-encoding DNA obtained from any biological cell, such as the cell of a mammal. Thus, the nucleic acids of the invention can be those of a mouse, rat, guinea pig, cow, sheep, horse, pig, rabbit, monkey, baboon, dog, or cat. Preferably, the nucleic acid molecules will be those of a human.

#### Expression of Mutant IL-15 Gene Products

The nucleic acid molecules described above can be contained within a vector that is capable of directing their expression in, for example, a cell that has been transduced with the vector. Accordingly, in addition to mutant IL-15 polypeptides, expression vectors containing a nucleic acid molecule encoding a mutant IL-15 polypeptide and cells transfected with these vectors are among the preferred embodiments.

degeneracy of the genetic code, encode the same polypeptide. These nucleic acid molecules can consist of RNA or DNA (for example, genomic DNA, cDNA, or synthetic DNA, such as that produced by phosphoramidite-based synthesis), or combinations or modifications of the nucleotides within these types of nucleic acids. In addition, the nucleic acid molecules can be double-stranded or single-stranded (i.e., either a sense or an antisense strand).

10 The nucleic acid molecules of the invention are referred to as "isolated" because they are separated from either the 5' or the 3' coding sequence with which they are immediately contiguous in the naturally occurring genome of an organism. Thus, the nucleic acid molecules  
15 are not limited to sequences that encode polypeptides; some or all of the non-coding sequences that lie upstream or downstream from a coding sequence can also be included. Those of ordinary skill in the art of molecular biology are familiar with routine procedures  
20 for isolating nucleic acid molecules. They can, for example, be generated by treatment of genomic DNA with restriction endonucleases, or by performance of the polymerase chain reaction (PCR). In the event the nucleic acid molecule is a ribonucleic acid (RNA),  
25 molecules can be produced by in vitro transcription.

The isolated nucleic acid molecules of the invention can include fragments not found as such in the natural state. Thus, the invention encompasses recombinant molecules, such as those in which a nucleic  
30 acid sequence (for example, a sequence encoding a mutant IL-15) is incorporated into a vector (for example, a plasmid or viral vector) or into the genome of a heterologous cell (or the genome of a homologous cell, at a position other than the natural chromosomal location).

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(SV40), and bovine papilloma virus vectors (see, for example, Gluzman (Ed.), *Eukaryotic Viral Vectors*, CSH Laboratory Press, Cold Spring Harbor, New York).

Prokaryotic or eukaryotic cells that contain and  
5 express a nucleic acid molecule that encodes a mutant IL-15 polypeptide are also features of the invention. A cell of the invention is a transfected cell, i.e., a cell into which a nucleic acid molecule, for example a nucleic acid molecule encoding a mutant IL-15 polypeptide, has  
10 been introduced by means of recombinant DNA techniques. The progeny of such a cell are also considered within the scope of the invention. The precise components of the expression system are not critical. For example, a mutant IL-15 polypeptide can be produced in a prokaryotic  
15 host, such as the bacterium *E. coli*, or in a eukaryotic host, such as an insect cell (for example, Sf21 cells), or mammalian cells (e.g., COS cells, NIH 3T3 cells, or HeLa cells). These cells are available from many sources, including the American Type Culture Collection  
20 (Rockville, MD). In selecting an expression system, it matters only that the components are compatible with one another. Artisans or ordinary skill are able to make such a determination. Furthermore, if guidance is required in selecting an expression system, skilled  
25 artisans may consult Ausubel et al. (*Current Protocols in Molecular Biology*, John Wiley and Sons, New York, NY, 1993) and Pouwels et al. (*Cloning Vectors: A Laboratory Manual*, 1985 Suppl. 1987).  
The expressed polypeptides can be purified from  
30 the expression system using routine biochemical procedures, and can be used as diagnostic tools or as therapeutic agents, as described below.

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Vectors suitable for use in the present invention include T7-based vectors for use in bacteria (see, for example, Rosenberg et al., *Gene* 56:125, 1987), the pMSXND expression vector for use in mammalian cells (Lee and Nathans, *J. Biol. Chem.* 263:3521, 1988), and baculovirus-derived vectors (for example the expression vector pBacPAK9 from Clontech, Palo Alto, CA) for use in insect cells. The nucleic acid inserts, which encode the polypeptide of interest in such vectors, can be operably linked to a promoter, which is selected based on, for example, the cell type in which expression is sought. For example, a T7 promoter can be used in bacteria, a polyhedrin promoter can be used in insect cells, and a cytomegalovirus or metallothionein promoter can be used in mammalian cells. Also, in the case of higher eukaryotes, tissue-specific and cell type-specific promoters are widely available. These promoters are so named for their ability to direct expression of a nucleic acid molecule in a given tissue or cell type within the body. Skilled artisans are well aware of numerous promoters and other regulatory elements which can be used to direct expression of nucleic acids.

In addition to sequences that facilitate transcription of the inserted nucleic acid molecule, vectors can contain origins of replication, and other genes that encode a selectable marker. For example, the neomycin-resistance (*neo<sup>r</sup>*) gene imparts G418 resistance to cells in which it is expressed, and thus permits phenotypic selection of the transfected cells. Those of skill in the art can readily determine whether a given regulatory element or selectable marker is suitable for use in a particular experimental context.

Viral vectors that can be used in the invention include, for example, retroviral, adenoviral, and adeno-associated vectors, herpes virus, simian virus 40



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polypeptide, including those that are a part of the chimeric polypeptides described above. This method may be used to treat a patient who is suffering from an autoimmune disease, including but not limited to the following: (1) a rheumatic disease such as rheumatoid arthritis, systemic lupus erythematosus, Sjögren's syndrome, scleroderma, mixed connective tissue disease, dermatomyositis, polymyositis, Reiter's syndrome or Behcet's disease (2) type II diabetes (3) an autoimmune disease of the thyroid, such as Hashimoto's thyroiditis or Graves' Disease (4) an autoimmune disease of the central nervous system, such as multiple sclerosis, myasthenia gravis, or encephalomyelitis (5) a variety of pemphigus, such as pemphigus vulgaris, pemphigus vegetans, pemphigus foliaceus, Senear-Usher syndrome, or Brazilian pemphigus, (6) psoriasis, and (7) inflammatory bowel disease (e.g., ulcerative colitis or Crohn's Disease). The administration of the mutant IL-15 polypeptide of the invention may also be useful in the treatment of acquired immune deficiency syndrome (AIDS). Similarly, the method may be used to treat a patient who has received a transplant of biological materials, such as an organ, tissue, or cell transplant. In addition, patients who have received a vascular injury would benefit from this method.

#### Induction of a Lytic Response

Through the administration of a lytic form of a mutant IL-15 chimeric polypeptide, it is possible to selectively kill autoreactive or "transplant destructive" immune cells without massive destruction of normal T-cells. Accordingly, the invention features a method of killing autoreactive or "transplant destructive" immune cells or any malignant cell that expresses the IL-15 receptor in vivo. The method is carried out by



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### Mutant IL-15 Polypeptides in Diagnosis

The polypeptide of the invention can be used to diagnose a patient as having a disease amenable to treatment with an IL-15 antagonist. According to this method, a sample of tissue is obtained from the patient and exposed to an antigenically-tagged mutant IL-15 polypeptide. The sample may be any biological sample, such as a blood, urine, serum, or plasma sample. In addition, the sample may be a tissue sample (e.g., biopsy tissue), or an effusion obtained from a joint (e.g. synovial fluid), from the abdominal cavity (e.g., ascites fluid), from the chest (e.g., pleural fluid), from the central nervous system (e.g., cerebral spinal fluid), or from the eye (e.g., aqueous humor). The sample may also consist of cultured cells that were originally obtained from a patient (e.g. peripheral blood mononuclear cells). It is expected that the sample will be obtained from a mammal, and preferably, that the mammal will be a human patient. If the sample contains cells that are bound by the polypeptide described (i.e., an antigenically-tagged mutant IL-15 polypeptide), it is highly likely that they would be bound by a mutant IL-15 polypeptide in vivo and thereby inhibited from proliferating, or even destroyed, in vivo. The presenting symptoms of candidate patients for such testing and the relevant tissues to be sampled given a particular set of symptoms are known to those skilled in the field of immunology.

### Modulation of the Immune Response

Also featured in the invention is a method of suppressing the immune response in a patient by administering a dose of mutant IL-15 that is sufficient to competitively bind the IL-15 receptor complex, and thereby modulate IL-15 dependent immune responses. The polypeptide administered can be a mutant IL-15

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oral administration. It is expected that the intravenous route will be preferred for the administration of mutant IL-15 polypeptides. The subcutaneous route may also be used frequently as the subcutaneous tissue provides a  
5 stable environment for the polypeptide, from which it can be slowly released.

The polypeptides of the invention are less likely to be immunogenic than many therapeutic polypeptides because they can differ from a wild-type polypeptide by  
10 only a few amino acid residues. In addition, the mutant IL-15 polypeptides are targeted to cells that express abundant receptors to which they can bind with high affinity.

It is well known in the medical arts that dosages  
15 for any one patient depend on many factors, including the general health, sex, weight, body surface area, and age of the patient, as well as the particular compound to be administered, the time and route of administration, and other drugs being administered concurrently. Dosages for  
20 the polypeptide of the invention will vary, but can, when administered intravenously, be given in doses of approximately 0.01 mg to 100 mg/ml blood volume. A dosage can be administered one or more times per day, if necessary, and treatment can be continued for prolonged  
25 periods of time, up to and including the lifetime of the patient being treated. If a polypeptide of the invention is administered subcutaneously, the dosage can be reduced, and/or the polypeptide can be administered less frequently. Determination of correct dosage for a given  
30 application is well within the abilities of one of ordinary skill in the art of pharmacology.

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administering to a patient an amount of mutant IL-15 linked to a polypeptide that is sufficient to activate the complement system, lyse cells by the ADCC mechanism, or otherwise kill cells expressing the wild-type IL-15 receptor complex. This method can be used to treat patients who have IL-15R<sup>+</sup> leukemia, lymphoma, or other IL-15R<sup>+</sup> malignant diseases, such as colon cancer.

#### Formulations for Use and Routes of Administration

In therapeutic applications, the polypeptide may be administered with a physiologically-acceptable carrier, such as physiological saline. The therapeutic compositions of the invention can also contain a carrier or excipient, many of which are known to skilled artisans. Excipients which can be used include buffers (for example, citrate buffer, phosphate buffer, acetate buffer, and bicarbonate buffer) amino acids, urea, alcohols, ascorbic acid, phospholipids, proteins (for example, serum albumin), EDTA, sodium chloride, liposomes, mannitol, sorbitol, and glycerol. The polypeptides of the invention can be formulated in various ways, according to the corresponding route of administration. For example, liquid solutions can be made for ingestion or injection; gels or powders can be made for ingestion, inhalation, or topical application. Methods for making such formulations are well known and can be found in, for example, "Remington's Pharmaceutical Sciences".

Routes of administration are also well known to skilled pharmacologists and physicians and include intraperitoneal, intramuscular, subcutaneous, and intravenous administration. Additional routes include intracranial (e.g., intracisternal or intraventricular), intraorbital, ophthalmic, intracapsular, intraspinal, intraperitoneal, transmucosal, topical, subcutaneous, and

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covalently bound to the 18 amino acid FLAG-HMK-sequence (FLAG-HMK-IL-15). FLAG sequences are recognized by biotinylated, highly specific anti-FLAG antibodies (Blanan et al., Science 256:1014, 1992); LeClair et al., Proc. Natl. Acad. Sci. USA 89:8145, 1992) while HMK (Heart Muscle Kinase recognition site) sequences allow introduction of radioactive label [<sup>32</sup>P] into the molecule (Blanan et al., supra, LeClair et al., supra).

For the construction of the plasmid FLAG-HMK-IL-15, a 322 bp cDNA fragment encoding mature IL-15 protein was amplified by PCR utilizing synthetic oligonucleotides [sense 5'-GGAATTCAACTGGGTGAATGTAATA-3' (SEQ ID NO:1; *EcoRI* site (underlined) plus bases 145-162); antisense 5'-CGGGATCCTCAAGAAGTGTTGATGAA-3' (SEQ ID NO:2; *BamHI* site 15'-[underlined] plus bases 472-489)]. The template DNA was obtained from PHA-activated human PBMCs. The PCR product was purified, digested with *EcoRI* and *BamHI*, and cloned into the pAR(DRI)59/60 plasmid digested with *EcoRI*-*BamHI* as described (Blanan et al., Science 256:1014, 1992; LeClair et al., Proc. Natl. Acad. Sci. USA 89:8145, 1992). The backbone of the pAR(DRI)59/60 plasmid contains in-frame sequences encoding the FLAG and HMK recognition peptide sequences (Blanan et al., Science 256:1014, 1992; LeClair et al., Proc. Natl. Acad. Sci. USA 89:8145, 1992).

#### Expression and Purification of FLAG-HMK-IL-15 Fusion Protein

The IL-15-related fusion construct, FLAG-HMK-IL-15, was expressed in BL-21 strain *E. coli* and affinity purified with anti-FLAG coated beads as described (Blanan et al., Science 256:1014, 1992; LeClair et al., Proc. Natl. Acad. Sci. USA 89:8145, 1992). The fusion protein was eluted from affinity columns after extensive washing with 0.1 M glycine (pH 3.0). The eluate containing FLAG-HMK-IL-15 was dialyzed against a buffer containing 50 mM

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EXAMPLESReagents

The following reagents were used in the studies described herein: recombinant human IL-2 was obtained from Hoffman-La Roche (Nutley, NJ); rapamycin was obtained from Wyeth-Ayerst (Princeton, NJ); cyclosporine-A (CsA) was obtained from Sandoz (East Hanover, NJ); RPMI-1640 and fetal calf serum (FCS) were obtained from BioWittaker (Walkersville, MD); penicillin, streptomycin, G418, and strepavidin-RED670 were obtained from Gibco-BRL (Gaithersburg, MD); dexamethasone, PHA, lysozyme, Nonidet P-40, NaCl, HEPES, and PMSF were obtained from Sigma (St. Louis, MO); Ficoll-Hypaque was obtained from Pharmacia Biotech (Uppsala, Sweden); recombinant human IL-15 and anti-human IL-15 Ab were obtained from PeproTech (Rocky Hill, NJ); anti-FLAG Ab and anti-FLAG-affinity beads were obtained from International Biotechnologies, Inc. (Kodak, New Haven, CT); pRcCMV was obtained from InVitrogen Corporation (San Diego, CA); genistein was obtained from ICN Biomedicals (Irvine, CA); disuccinimidyl suberate (DSS) was obtained from Pierce (Rockford, IL); restriction endonucleases were obtained from New England Biolabs (Beverly, MA); [<sup>3</sup>H]TdR was obtained from New England Nuclear (Boston, MA); and fluorescent dye conjugated antibodies CD25-PE<sup>3</sup>, CD14-PE, CD16-PE, CD122-PE, CD4-FITC, CD8-FITC, IgG1-PE or IgG1-FITC were obtained from Beckton/Dickinson (San Jose, CA). FLAG peptide was synthesized in the Peptide Synthesis Facility at Harvard Medical School.

30

Production of FLAG-HMK-IL-15 Fusion Protein

To study the cellular pattern of human IL-15 receptor expression, a plasmid that could be used to express an IL-15 fusion protein was constructed. The plasmid encodes an IL-15 polypeptide having an N-terminus



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For studies involving rapamycin or genistein, human PBMCs ( $4 \times 10^5$ /well) were plated in U-bottomed 96-well tissue culture plates (NUNC; Naperville, IL) with rapamycin (10 ng/ml), genistein (10  $\mu$ g/ml) or media alone, and cultured for 3 days at 37°C and 5% CO<sub>2</sub>. Cell viability was tested utilizing the standard trypan blue exclusion method (Current Protocols in Immunology, Vol. 3, ed. R. Coico, New York: John Wiley & Sons, 1995).

#### BAF-BO3 Cells

10 The BAF-BO3 cell line is an IL-3-dependent, murine pro-B cell line. A BAF-BO3 cell line that was pre-selected for high expression of the IL-2R $\alpha$  subunit was kindly provided by T. Taniguchi (Osaka, Japan; Hatakeyama et al., 1989, Cell 59:837). BAF-BO3 cells were  
15 transfected with vector control (pRcCMV-0) or cDNA encoding the human IL-2R $\beta$  subunit cloned into pRcCMV (pRcCMV-IL-2R $\beta$ ), by electroporation at 350 volts and 500  $\mu$ Farads using 20  $\mu$ g of ScaI linearized plasmid. Transfected cells were selected in the presence of 1  
20 mg/ml G418 and tested for the expression of the IL-2R $\beta$  subunit by fluorescence activated cell sorting (FACS) analysis as described (Maslinski et al., Int. Immunol. 4:509, 1992; Hatakeyama et al., Cell 59:837, 1989).

BAF-BO3 cells were maintained at 37°C with 5% CO<sub>2</sub>  
25 in RPMI-1640 medium supplemented with 10% FCS, penicillin (0.5 U/ml), streptomycin (0.5  $\mu$ g/ml), and 5% (V/V) IL-3 rich WEHI cell supernatant (Hatakeyama et al., Cell 59:837, 1989).

To test mutant polypeptides, a cytotoxic  
30 T lymphocyte cell line, CTLL-2, can also be used. This cell line is available from the American Type Culture Collection (12301 Parklawn Drive, Rockville, MD 20852-1776).



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Tris (pH 7.4) and 0.1 M NaCl for 18 hours at 4°C, filtered through a 0.2 µm membrane, and stored at -20°C.

#### Analysis of FLAG-HMK-IL-15 Fusion Protein

The authenticity of FLAG-HMK-IL-15 fusion protein was confirmed as follows. The proteins present in eluates from the affinity column were separated by SDS-PAGE, transferred to PVDF membranes, and stained with Coomassie blue (Fig. 1, lane a). The presence of FLAG sequences was specifically examined using anti-FLAG Ab (Fig. 1, lane b), and the presence of hIL-15 sequences was specifically examined using anti-hIL-15 Ab (Fig. 1, lane c). A 15 kDa protein, whose mass corresponds to the expected size of FLAG-HMK-IL-15 protein, was identified in each of these analyses. To confirm that the fusion protein contains the HMK recognition site, eluates from the affinity column were incubated in the presence of enzymatically active HMK and radioactive [<sup>32</sup>P]-γ-ATP, followed by separation of proteins by SDS-PAGE.

Autoradiography again identified a single radiolabeled 15 kDa band that co-migrates with the FLAG-HMK-IL-15 (Fig. 1, lane d). Thus, the 15 kDa protein contains all three elements of the designed fusion protein: FLAG sequence, HMK sequence, and hIL-15 sequence.

#### Cell Culture

##### Human PBMCs

Human peripheral blood mononuclear cells (PBMCs) were obtained from leukaphoresis preparations from healthy donors and isolated by Ficoll-Hypaque density gradient centrifugation according to standard protocols. PBMCs were maintained at 37°C in an atmosphere containing 5% CO<sub>2</sub> in RPMI-1640 medium supplemented with 10% FCS, penicillin (0.5 U/ml), and streptomycin (0.5 µg/ml).

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subunits, and which, therefore, proliferate in response to exogenously added human IL-2 or IL-15 (Giri et al., EMBO J. 13:2822, 1994). Cells were washed twice to remove the medium containing the growth factor IL-3, and  
5 starved for 16 hours in RPMI-1640 medium supplemented with 10% FCS, penicillin (0.5 U/ml), and streptomycin (0.5 µg/ml). Cells were then plated at  $2 \times 10^4$  cells/well, stimulated with FLAG-HMK-IL-15, and cultured for 72 hours at 37°C in an atmosphere containing 5% CO<sub>2</sub>.  
10 This was followed by a 14-hour "pulse" with 1 µCi of [<sup>3</sup>H]-TdR. The BAF-BO3 cells were then harvested and cell-associated radioactivity was measured by scintillation counting as described above.

15 Cross-linking Activated PBMCs Cultured with FLAG-HMK-IL-15

After 72 hours in culture, PHA-activated PBMCs were washed twice in cold phosphate-buffered saline (PBS), and  $10^7$  cells/ml were incubated for 30 minutes at 4°C in 400 µl of RPMI-1640 medium supplemented with 25 mM  
20 HEPES (pH 7.4), 10% FCS, penicillin (0.5 U/ml), and streptomycin (0.5 µg/ml). Supplemented RPMI-1640 medium (20 µl) containing 1 µg of recombinant human IL-15 as a control for non-specific binding or medium alone was then added, and the incubation was continued for 15 minutes  
25 before 20 µl of medium containing [<sup>32</sup>P]-labeled FLAG-HMK-IL-15 (1 ng, 500,000 cpm) was added to each sample. After further incubation for 1 hour at 4°C, the cells were washed with PBS and resuspended in 250 µl of PBS. Disuccinimidyl suberate (DSS; 1 mM), a cross-linker, was  
30 added, and cross-linking was performed as described by Tsudo et al. (Proc. Natl. Acad. Sci. USA 83:9694, 1986). The cross-linked cells were collected by centrifugation, washed, solubilized with extraction buffer (2% Nonidet P-40, 0.14 M NaCl, 25 mM HEPES (pH 7.4), 1 mM phenyl-  
35 methylsulfonyl fluoride) and centrifuged. Proteins

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Cellular Proliferation AssaysHuman PBMCs

Peripheral blood mononuclear cells ( $1 \times 10^6/\text{ml}$ ) were stimulated with PHA ( $2 \mu\text{g}/\text{ml}$ ) for 72 hours at  $37^\circ\text{C}$  in RPMI-1640 medium supplemented with 10% FCS, penicillin, and streptomycin, as described above. To investigate the effect of immunosuppressive drugs, aliquots of cells ( $10^6$  PBMCs/ml) were preincubated with cyclosporin-A (CsA;  $150 \text{ ng}/\text{ml}$ ), rapamycin ( $5 \text{ ng}/\text{ml}$ ), or dexamethasone ( $0.1 \text{ mg}/\text{ml}$ ) for 20 minutes before the addition of PHA.

In some experiments, cells prepared as described above were washed after 72 hours in culture, plated in U-bottomed 96-well tissue culture plates at  $2 \times 10^5$  PBMCs per well (NUNC, Naperville, IL), and then re-stimulated with one of the following reagents: PHA ( $2 \mu\text{g}/\text{ml}$ ), FLAG peptide ( $10^{-4} \text{ M}$ ), IL-2 ( $100 \text{ U}/\text{ml}$ ), or FLAG-HMK-IL-15 ( $100 \text{ ng}/\text{ml}$ ) for 72 hours. Cells were "pulsed" for 4 hours with  $1 \mu\text{Ci}$  of [ $^3\text{H}$ ]Tdr (New England Nuclear, Boston, MA), harvested onto Whatman 934-AH glass microfiber filters, and hypotonically lysed using a PHD Cell Harvester (Cambridge Technology, Inc., Cambridge, MA). Cell associated [ $^3\text{H}$ ]Tdr was measured using a Beckman LS 2800 scintillation counter (Beckman, Fullerton, CA).

25 BAF-BO3 cells

IL-3 dependent BAF-BO3 cells, which constitutively express the IL-2R $\gamma$  subunit, were transfected with a control pRcCMV construct (pRcCMV-0) or a pRcCMV construct containing cDNA encoding the full-length human IL-2R $\beta$  subunit and the neo<sup>r</sup> gene (pRcCMV-IL-2R $\beta$ ). G418 resistant clones were then selected. Cells that were selected in this way were used to test the biological activity of FLAG-HMK-IL-15. This strategy has been used to identify cells transfected with cDNA encoding full-length IL-2R $\beta$ .

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molecular weight of FLAG-HMK-IL-15 (15 kDa) and the human IL-15R $\alpha$  subunit (60-65 kDa; Fig. 2, lane 1). The identity of this band as the IL-15R $\alpha$  subunit was confirmed by cross-linking experiments conducted in the presence of a molar excess of hIL-15. Under these conditions, we failed to detect the radiolabeled 15 kDa band (Fig. 2, lane 2). Thus, the conformation of [<sup>32</sup>P]-FLAG-HMK-IL-15 fusion proteins allows site specific binding to the 60-65 kDa IL-15R $\alpha$  subunit expressed on the surface of mitogen-activated PBMCs.

FLAG-HMK-IL-15 is a Biologically Active Growth Factor that Requires Expression of IL-2R $\beta$

In the next series of experiments, the FLAG-HMK-IL-15 fusion protein was tested in order to determine whether it could function as a biologically active growth factor. PHA-activated human PBMCs proliferate in response to either FLAG-HMK-IL-15 or human recombinant IL-2, as detected via the [<sup>3</sup>H]-TdR incorporation assay described above. A FLAG peptide lacking the IL-15 sequence does not stimulate cell proliferation (Fig. 3a). As does IL-2, the FLAG-HMK-IL-15 fusion protein stimulates proliferation of IL-2R $\gamma$ BAF-B03 cell transfectants that express the IL-2R $\beta$  subunit (Fig. 3b, right-hand panel). The FLAG-HMK-IL-15 fusion protein does not, however, stimulate the proliferation of parental BAF-B03 cells that were transfected with a vector lacking IL-2R $\beta$  chain sequences (Fig. 3b, left-hand panel). Thus, FLAG-HMK-IL-15 is a biologically active growth factor that requires expression of IL-2R $\beta$  chains upon target cells in order to stimulate cellular proliferation.

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present in the supernatants of these cell lysates were separated by 8% SDS-PAGE under reducing conditions. An autoradiogram was developed from the dry gel after exposure at -70°C overnight.

5           Cell staining for flow cytometry

Resting or PHA-activated PBMCs ( $3 \times 10^5$  cells/tube) were washed twice with ice-cold PBS/0.02% sodium azide and incubated in medium containing FLAG-HMK-IL-15 ( $0.1 \mu\text{g}/100 \mu\text{l}$ ) or, as a control, in medium alone. 10 The incubation was carried out on ice for 30 minutes. The cells were then washed with PBS and incubated for 2 sequential 30-minute periods at 4°C with biotin-conjugated anti-FLAG monoclonal antibody ( $0.5 \mu\text{g}/100 \mu\text{l}$ ) and streptavidin-RED670 ( $0.5 \mu\text{g}/100 \mu\text{l}$ ). The cells were 15 then counterstained with CD25-PE, CD14-PE, CD16-PE, CD122-PE, CD4-FITC, or CD8-FITC, as indicated. Addition of IgG1-PE or IgG1-FITC was added to cell populations that had not been incubated with any of the aforementioned reagents in order to identify non-specific 20 binding. Cell surface phenotype was analyzed using FACScan (Becton/Dickinson, CA) and Cell Quest software.

FLAG-HMK-IL-15 binds the IL-15R $\alpha$  subunit

The purified FLAG-HMK-IL-15 fusion protein was tested to determine whether it interacts with cell 25 surface IL-15 receptors. As described above, [ $^{32}\text{P}$ ]-FLAG-HMK-IL-15 was added to cultures of PBMCs that were activated by a mitogen, PHA. In order to permanently bind interactive proteins to one another, the chemical cross-linker disuccinimidyl suberate (DSS) was added. 30 The cells were washed, lysed, centrifuged, and detergent-soluble proteins were separated by SDS-PAGE. Autoradiography of SDS-PAGE separated proteins revealed a single 75-80 kDa band corresponding to the combined



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positive) were noted (Fig. 5B). Interestingly, there are IL-2R $\beta$  cells that fail to bind IL-15 (Fig. 5B). Therefore, expression of IL-2R $\beta$  chains is not sufficient for IL-15 binding.

5 Taken together, these data indicate that IL-15 can bind IL-15R $\alpha$ , IL-2R $\alpha$ , IL-2R $\beta$  cells. A similar conclusion was reached through experimentation that probed the interaction of IL-15 with IL-2R $\alpha$ ,  $\beta$  cells transfected with IL-15R $\alpha$  subunit (Anderson et al., J. Biol. Chem. 270:29862, 1995; Giri et al., EMBO J. 14:3654, 1995).

10 In addition to the requirement for IL-15R $\alpha$  subunit expression, the IL-2R $\beta$  and IL-2R $\gamma$  subunits are required to render cells sensitive to IL-15 triggered growth (Fig. 3, see also Fig. 5).

Dexamethasone, but not Cyclosporine or Rapamycin Blocks Mitogen-induced Expression of the IL-15R $\alpha$  Subunit

The effects of several immunosuppressive drugs on PHA-induced expression of IL-15R $\alpha$  by PBMCs were also studied. The drugs tested were cyclosporine-A (CsA), rapamycin, and dexamethasone. The addition of CsA to PBMCs cultured with PHA resulted in a 20% reduction in the expression of IL-2R $\alpha$ , but IL-15R $\alpha$  was unaffected (Fig. 6). The addition of rapamycin actually resulted in enhanced IL-15R $\alpha$  expression (Fig 6C). In contrast to the effects of CsA and rapamycin, dexamethasone powerfully blocked the expression of IL-15R $\alpha$  chains on mitogen activated PBMCs (Fig. 6D). In accordance with these data, PHA-activated, dexamethasone-treated cells proliferated poorly in response to IL-15, while the response to IL-2 was not inhibited by dexamethasone (Fig. 7). In this experiment, PMBCs were cultured in the presence of PHA and various immuno-suppressive drugs, and then washed and cultured further in the presence of

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Mitogen-activated, but not Resting, PBMCs Express the IL-15R $\alpha$  Subunit

The FLAG-HMK-IL-15 fusion protein, biotinylated anti-FLAG antibody, and streptavidin-RED670 were employed to detect expression of IL-15 binding sites on human PBMCs by cytofluorometric analysis. The PBMCs tested were either freshly isolated or PHA-activated. These cells were washed and incubated with either medium alone or FLAG-HMK-IL-15 followed by anti-FLAG biotinylated Ab and streptavidin-RED670. The stained cells were analyzed by flow cytometry (Fig. 4A). PBMCs that were activated with PHA expressed IL-15R $\alpha$  proteins but resting PBMCs did not. In keeping with the result of the cross-linking experiments described above (Fig. 2), binding of FLAG-HMK-IL-15 to PHA activated PBMCs is blocked by a molar excess of rIL-15 (Fig. 4B), thereby demonstrating the specificity of FLAG-HMK-IL-15 binding for IL-15 binding sites. Both activated CD4<sup>+</sup> and CD8<sup>+</sup> cells express IL-15 $\alpha$  chains (Fig. 4C). Activation induced IL-15R $\alpha$  chains were also detected on CD14<sup>+</sup> (monocyte/macrophage) cells and CD16<sup>+</sup> (natural killer) cells.

IL-2R $\alpha$  and IL-2R $\beta$  Subunits Are Not Required for IL-15 Binding

FACS analysis of PHA-activated PBMCs stained with FLAG-HMK-IL-15 proteins and anti-CD25 Mab, against the IL-2R $\alpha$  subunit, reveals cell populations expressing both IL-15R $\alpha$  and IL-2R $\alpha$  subunits, as well as cell populations that express either subunit, but not both (Fig. 5A). There are IL-2R $\alpha$ <sup>+</sup> cells that do not bind FLAG-HMK-IL-15 (Fig. 5A). Almost all PBMCs that were stimulated with PHA for only one day express either IL-15R $\alpha$  or IL-2R $\beta$  chains, but not both proteins (Fig. 5B). In contrast, 3 days following PHA stimulation, a far larger population of IL-15R $\alpha$ <sup>+</sup>, IL-2R $\beta$ <sup>+</sup> cells (double positive) and a far smaller population of IL-15R $\alpha$ <sup>+</sup>, IL-2R $\beta$ <sup>-</sup> cells (single

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results, in conjunction with the data showing CsA-independent induction of IL-15 gene expression, suggest that CsA may not block IL-15 triggered immune responses. On the contrary, efficiently blocking the induction of the IL-15R $\alpha$  subunit with dexamethasone may be very helpful in preventing IL-15 induced cellular proliferation.

In summary, the experiments presented above have demonstrated that: (i) IL-15R $\alpha$  subunits are rapidly expressed by activated macrophages, T cells, and NK cells, and (ii) induction of the IL-15R $\alpha$  subunit is blocked by dexamethasone but not by CsA or rapamycin. In addition, the experiments have confirmed that the IL-15R $\alpha$  subunit is necessary and sufficient for IL-15 binding and that the FLAG-HMK-IL-15 fusion protein is an extremely useful tool for studying IL-15 receptors.

#### A COMPARATIVE ANALYSIS OF THE IL-2 AND IL-15 SIGNALLING PATHWAYS

In this series of experiments, the intracellular signalling pathway that is initiated when IL-2 binds to the IL-2R $\alpha$ , - $\beta$ , - $\gamma$  receptor complex was compared to the pathway initiated when IL-15 binds to the receptor complex composed of IL-15R $\alpha$ , IL-2R $\beta$ , and IL-2R $\gamma$ .

The methods, in addition to those described above, required to perform these experiments follow.

#### Cell Culture, Cell Lysis and Protein Immunoblotting

Fresh human PBMCs were isolated as described above and cultured in RPMI-1640 medium supplemented with 10% FCS, penicillin, and streptomycin (also as above). To achieve activation by a mitogen, PHA (10  $\mu$ g/ml) was added to the culture for 3 days. PHA-stimulated PBMCs were washed 3 times with PBS, cultured for 14 hours in RPMI-1640 medium containing 10% FCS, penicillin, and

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medium alone ("none"), PHA, IL-2, or IL-15 for 2 days before a [<sup>3</sup>H]-TdR pulse. The potent response of PHA-activated, dexamethasone-treated PBMCs to IL-2 proves that the ability of dexamethasone to inhibit the proliferative response to IL-15 is not due to a non-specific toxic effect.

The studies described above used FLAG-HMK-IL-15 and flow cytometry analysis to determine whether resting and mitogen-activated PBMCs express the IL-15R $\alpha$  subunit. The IL-15R $\alpha$  subunit is rapidly expressed by activated but not resting lymphocytes, NK-cells, and macrophages (Fig. 4).

These studies have also shown that the induction of IL-15R $\alpha$  by mitogen is sensitive to an immunosuppressant: PBMCs pre-treated with dexamethasone and stimulated with PHA do not express IL-15R $\alpha$  as vigorously as cells that were not pre-treated with dexamethasone (Fig. 6). Similarly, PBMCs pre-treated with dexamethasone do not proliferate as robustly in response to exogenously added IL-15 (Fig. 7). The cells do respond partially to IL-15. This may reflect that fact that IL-15R $\alpha$  is expressed within 24 hours of dexamethasone withdrawal. CsA, a potent inhibitor of IL-2 and IL-2R $\alpha$  expression (Farrar et al., J. Biol. Chem. 264:12562, 1989), is ineffective in preventing the mitogen-induced expression of the IL-15R $\alpha$  chain (Fig. 6). There was a slight reduction in the proliferative response to exogenously added IL-15 among cells pre-treated with CsA for 72 hours (Fig. 7), however this reduction may be due to inhibition of any number of events in the CsA-sensitive, Ca<sup>2+</sup>-dependent pathway of T cell activation. Since the same modest reduction in cell proliferation was observed in response to IL-2, CsA may exert certain long lasting effects by blocking signaling pathway(s) shared by IL-2 and IL-15. These

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propagates cells that are equally sensitive to further stimulation with either cytokine. Hence, according to the scheme shown in Fig. 8A, stimulation of mitogen-activated PBMCs with IL-2 or IL-15 does not  
5 select for a bulk cell population that then responds only to IL-2 or only to IL-15.

Further experiments were carried out using T cells pre-stimulated with PHA for 3 days followed by stimulation with IL-2 for 2 days. IL-2 stimulated cell  
10 proliferation is a protein tyrosine kinase dependent event (Maslinski et al. *J. Biol. Chem.* 267:15281, 1992); Remillard et al., *J. Biol. Chem.* 266:14167, 1991). Thus, an inhibitor of tyrosine kinases, genistein, was used to test whether the proliferative signals induced by IL-15  
15 are mediated by the same tyrosine kinases as are the proliferative signals induced by IL-2. IL-2 and IL-15 both induce T cell proliferation and both manifest a similar dose-related sensitivity to genistein (Fig. 9).

To rule out the possibility that genistein was  
20 functioning as a general toxin, rather than a selective inhibitor of protein kinase-dependent IL-2 or IL-15-induced cell proliferation, a standard trypan blue exclusion assay was performed. Cell viability after 3 days of incubation in the presence of genistein  
25 (10  $\mu$ g/ml) was 65%  $\pm$  15% of the control (where no genistein was added). Therefore, most of the observed inhibitory effect of genistein is due to inhibition of IL-2 and IL-15 triggered signal transduction.

Tyrosine phosphorylation events are critical for  
30 IL-15 stimulated proliferation. Indeed, a direct comparison of the pattern of tyrosine phosphorylation shows that the protein phosphorylation induced by IL-2 is identical to the protein phosphorylation induced by IL-15 (Fig. 10). This indicates that both cytokines stimulate  
35 similar, if not identical, protein tyrosine kinases.



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streptomycin, then cultured in medium containing recombinant human IL-2 (100 U/ml), recombinant human IL-15 (10 ng/ml) or non-supplemented medium, for 10 minutes at 37°C.

- 5 After stimulation with interleukins, the cells were washed in cold PBS and lysed in an ice-cold lysis buffer (150 mM NaCl, 20 mM Tris (pH 7.5), 1 mM phenylmethylsulfonyl fluoride, 0.5 mg/ml leupeptin, 10 mg/ml aprotinin, 1 mM Na<sub>2</sub>VO<sub>4</sub>, 50 mM NaF, and 1% NP-40).
- 10 Lysates were incubated for 15 minutes on ice and then pelleted at 100,000 x g for 30 minutes.

Soluble proteins were separated by SDS-PAGE (10% acrylamide) followed by transfer to a PVDF membrane (Millipore, Bedford, MA). The membrane was then incubated in blocking buffer (25 mM HEPES (pH 7.4), 150 mM NaCl, 0.05% Tween, and 2% BSA) for 2 hours at room temperature, followed by incubation with an anti-phosphotyrosine antibody, RC-20, conjugated to alkaline phosphate (Signal Transduction Labs, Inc. Lexington, KY) for 2 hours at room temperature. Washed blots were developed in the BCIP/NBT phosphatase substrate solution for colorimetric analysis (Kirkegard and Perry Laboratories, Inc. Gaithersburg, MD).

25 The pattern of Tyrosine Phosphorylated Proteins Induced by IL-2 is the Same as that Induced by IL-15

To select a population of activated, IL-2 responsive T cells, PBMCs were stimulated with PHA for 3 days, washed, and then stimulated with IL-2 or IL-15 for an additional 2 days. Cultivation of lymphocytes in IL-2 rich medium leads to propagation of lymphocytes that express a high copy number of the tri-molecular high-affinity IL-2R complex (Maslinski et al., J. Biol. Chem. 267:15281, 1992). Furthermore,

35 cultivation of PHA-treated PBMCs with IL-2 or IL-15

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The Immunosuppressant Cyclosporin does not Inhibit  
Either IL-2 or IL-15 Induced Cellular  
Proliferation

The effect of a second immunosuppressant, cyclosporine-A (CsA), on IL-15 induced cell proliferation was also examined. Cyclosporine-A is a peptide that forms complexes with cyclophilin; these complexes bind to and inhibit the enzymatic activity of the cellular phosphate calcineurin, resulting, among other effects, in blockade of IL-2 and IL-2R $\alpha$  gene transcription (Sigal et al., *Annu. Rev. Immunol.* 10:519, 1992). T cells expressing the tri-molecular IL-2R complex and stimulated with exogenously added IL-2 are not sensitive to CsA (Sigal et al., *Annu. Rev. Immunol.* 10:519, 1992). It has now been shown that CsA does not inhibit IL-15 or IL-2 triggered cellular proliferation (Fig. 11), again revealing similarities between IL-2 and IL-15 induced cellular proliferation.

The doses of CsA used in these studies (to block PHA-induced IL-2 gene expression) were tested in order to provide evidence that a sufficient amount of CsA was being used. CsA (100 ng/ml) completely blocked PHA-induced IL-2 mRNA expression as judged by the results of RT-PCR and competitive PCR techniques, as described before (Lipman et al., *J. Immunol.* 152:5120, 1994). This result confirms the efficacy of CsA in the culture system used herein. Moreover, it discounts the possibility that IL-15 induces IL-2 gene expression that, in turn, could be responsible for T cell proliferation.

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The IL-2R $\beta$  Subunit is Critical for both IL-2 and  
IL-15 Signal Transduction

The hypothesis that elements of the IL-2R $\beta$  chain that are required for IL-2 signal transduction are also required for IL-15 signal transduction was tested. Previous studies indicated that BAF-BO3 cells that

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IL-2 and IL-15 Stimulated Proliferative Events are Equally Sensitive to Inhibition by Rapamycin

To further characterize the signaling events that are mediated by IL-2 and IL-15, the sensitivity of IL-2 and IL-15 induced T cell proliferation to rapamycin, which inhibits IL-2R signal transduction. Rapamycin, a macrolide with potent immunosuppressive activity (Sigal et al., *Annu. Rev. Immunol.* **10**:519, 1992; Sehgal et al., *ASHI Quarterly* **15**:8, 1991), inhibits IL-2 induced signal transduction prior to or at the level of activation of p70 S6 kinase and cyclin E dependent cdk2 kinase (Price et al., *Science* **257**:973, 1992; Bierer et al., *Proc. Natl. Acad. Sci. USA* **87**:9231, 1990; Calvo et al., *Proc. Natl. Acad. Sci. USA* **89**:7571, 1992; and Chung et al., *Cell* **69**:1227, 1992). PHA-stimulated, IL-2-activated cells were washed prior to incubation with rapamycin and cultured with IL-2 or IL-15. Again, the similarity of IL-2 and IL-15 induced T cell proliferative events was evident: the proliferative events stimulated by these two interleukins were equally dose-sensitive to inhibition by rapamycin (Fig. 10). Just as in studies involving genistein (see above), the possibility that rapamycin was toxic to lymphocytes was ruled out by performing standard trypan blue exclusion assays (*Current Protocols in Immunology*, Vol. 3, ed: Coico, R. New York: John Wiley & Sons, 1995). Cell viability after three days of incubation with rapamycin (10 ng/ml) was  $8.5\% \pm 10\%$  of the control, where no drug was added. Therefore, the observed inhibition of cellular proliferation by rapamycin is due to an inhibition of IL-2 and IL-15 induced signal transduction.

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relatively early events in the course of IL-2 and IL-15 intracellular signal transduction, the terminal phase of IL-2 and IL-15 signal transduction are also likely to be quite similar insofar as activation of target cells with IL-2 or IL-15 gives rise to expression of the same DNA binding proteins (Giri et al., *EMBO J.* 13:2822, 1994). These results also suggest that the function of the IL-15R $\alpha$  and IL-2R $\alpha$  chain are similar, i.e. that they are most important for cytokine binding affinity and have a negligible role in signal transduction.

Decreasing the viability of activated T cells or blocking the signal transduction pathways activated by IL-2 and IL-15 provides a way to decrease the production of lymphokines and mitogens that contribute to accelerated atherosclerosis, allograft rejection, certain leukemias and other immune-mediated pathologies. When activated, T cells proliferate and express receptors on their cell surface for interleukins. In addition, activated T cells release at least 3 lymphokines: gamma interferon, B cell differentiation factor II, and IL-3. These lymphokines can produce various undesirable events, such as allograft rejection. In contrast, resting T cells and long-term memory T cells do not express lymphokine receptors. This difference in receptor expression provides a means to target activated immune cells without interfering with resting cells. Molecules designed to recognize some subunit of the IL-15R will recognize activated monocytes/macrophages as well as activated T cells and can be used to selectively inhibit or destroy these cells. Derivatives of IL-15 that bind to an IL-15R subunit but that lack IL-15 activity, either because they block the binding and/or uptake of bona fide IL-15, are useful in the method of the invention. The mutant IL-15 molecule described below provides a working example of such a derivative.

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express endogenous IL-2R $\alpha$  and IL-2R $\gamma$  subunits can be rendered IL-2 dependent upon transfection with cDNA encoding human the IL-2R $\beta$  protein (Hatakeyama et al., Science 244:551, 1989). In contrast, BAF-BO3 cells that  
5 express a mutant IL-2R $\beta$  protein, which lacks a 71 amino acid serine-rich region, do not respond to IL-2 (Hatakeyama et al., Science 252:1523, 1991; Hatakeyama et al., Science 244:551, 1989; and Fung et al., J. Immunol. 147:1253, 1991). Therefore, the ability of IL-15 to  
10 induce the proliferation of IL-2R $\alpha\gamma$  BAF-BO3 cells that express either the wild-type IL-2R $\beta$  subunit or a mutant IL-2R $\beta$  protein that lacks the serine rich region (S). While both IL-2 and IL-15 stimulate the proliferation of cells that express the wild-type IL-2R $\beta$  subunit, neither  
15 cytokine is able to stimulate IL-2R $\alpha\gamma$  BAF-BO3 cells that express mutant S IL-2R $\beta$  subunit (Fig. 12). These results demonstrate that the serine-rich region of the IL-2R $\beta$  subunit is critical for both IL-2 and IL-15 triggered signal transduction.

20 The serine-rich region of the IL-2R $\beta$  subunit binds several tyrosine kinases including Jak-1 and Syk (Minami et al., Immunity 2:89, 1995). The same kinases may play a critical role in both IL-2 and IL-15 signal transduction. Since BAF-BO3 cells cannot express IL-2  
25 (Anderson et al., J. Biol. Chem. 270:29862, 1995), even upon stimulation with IL-15, these results indicate that IL-15 induced cellular proliferation does not require induction of IL-2 gene expression.

The experiments performed in this series (see also  
30 Lin et al., Immunity 2:331, 1995; Anderson et al., J. Biol. Chem. 270:29862, 1995) indicate that IL-2 and IL-15 triggered signal transduction use overlapping, perhaps identical, signaling pathways, and that agents that block IL-2 signaling are highly likely to block IL-  
35 15 signaling. Although these experiments compared



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Similarly, this strategy can be used to incorporate any other amino acid in place of the glutamine residues at positions 149 or 156 or to introduce amino acid substitutions at positions other than 149 and/or 156.

Proliferation of BAF-BO3 Cells in the Presence of IL-15 Related Proteins

In order to study the effect of various IL-15 related proteins, including the mutant polypeptides described above, on the proliferation of BAF-BO3 cells, the following experiment was performed in vitro. BAF-BO3 cells were transfected in culture with DNA from either pRCCMV-IL-2R $\beta$  or pRCCMV-0. These plasmids differ in that pRCCMV-IL-2R $\beta$  contains an insert encoding the human IL-15 2R $\beta$  subunit and pRCCMV-0 does not. Following transfection, the cells were washed and treated with either: (1) unsupplemented medium ("none"), (2) IL-2, (3) IL-3 rich WEHI cell supernatant (WEHI), (4) FLAG-HMK-IL-15, (5) FLAG-HMK-IL-15 Q149D single mutant (149), (6) FLAG-HMK-IL-15 Q156D double mutant (DM), (7) IL-15, or (8) IL-15 + FLAG-HMK-IL-15 Q149D Q156D double mutant (Figs 15). Cells transfected with pRCCMV-0 DNA, did not proliferate in response to any stimulus except WEHI cell supernatant. In contrast, following transfection with pRCCMV-IL-2R $\beta$  DNA, IL-2, WEHI cell supernatant, FLAG-HMK-IL-15, FLAG-HMK-IL-15 Q149D single mutant, and IL-15 stimulated cellular proliferation. The IL-2R $\beta$  expressing cells did not proliferate in response to the double mutant IL-15. The double mutant IL-15 polypeptide may inhibit BAF-BO3 proliferation in a dose-dependent manner: addition of 30  $\mu$ l (approximately 50  $\mu$ g/ml) of the double mutant IL-15 inhibited proliferation more completely than did addition of 20  $\mu$ L of the same concentration of the double mutant IL-15.

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A MUTANT IL-15 POLYPEPTIDE THAT FUNCTIONS AS AN  
ANTAGONIST OF WILD-TYPE IL-15

Genetic Construction of mutant IL-15

The human IL-15 protein bearing a double mutation  
5 (Q149D; Q156D) was designed to target the putative sites  
critical for binding to the IL-2R $\gamma$  subunit. The polar,  
but uncharged glutamine residues at positions 149 and 156  
(Fig. 12) were mutated into acidic residues of aspartic  
acid (Fig. 13) utilizing PCR-assisted mutagenesis. A  
10 cDNA encoding the double mutant of IL-15 was amplified by  
PCR utilizing a synthetic sense oligonucleotide  
[5'-GGAATTCAACTGGGTGAATGTAATA-3' (SEQ ID NO:1); *EcoRI* site  
(underlined hexamer) plus bases 145-162] and a synthetic  
antisense oligonucleotide  
15 [5'-CGGGATCCTCAAGAAGTGTTGATGAACATGTCGACAATATGTACAAAACTGTC  
CAAAAAT-3' (SEQ ID NO:3); *BamHI* site (underlined hexamer)  
plus bases 438-489; mutated bases are singly underlined].  
The template was a plasmid containing cDNA that encodes  
human FLAG-HMK-IL-15. The amplified fragment was  
20 digested with *EcoRI/BamHI* and cloned into the  
PAR(DRI) 59/60 plasmid digested with *EcoRI/BamRI* as  
described (LeClair et al., *Proc. Natl. Acad. Sci. USA*  
89:8145, 1989). The presence of a mutation at residue  
156 was confirmed by digestion with *SalI*; the mutation  
25 introduces a new *SalI* restriction site. In addition,  
mutations were verified by DNA sequencing according to  
standard techniques. The FLAG-HMK-IL-15 (Q149D; Q156D)  
double mutant protein was produced, purified, and  
verified by sequencing as described above for the FLAG-  
30 HMK-IL-15 wild-type protein.

Using this same strategy, mutants that contain  
only a single amino acid substitution, either at position  
149 or at position 156 were prepared. As described  
above, these positions (149 and 156) correspond to  
35 positions 101 and 108, respectively, in the mature IL-15  
polypeptide, which lacks a 48-amino acid signal sequence.

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### Genetic Construction of Additional Mutant IL-15 Chimeric Polypeptides

In addition to the FLAG-HMK-IL-15 chimera, which provides the mutant IL-15 with an antigenic tag, numerous other polypeptides can be linked to any mutant of IL-15. For example, mutant IL-15 can be linked to serum albumin or to the Fc fragment of the IgG subclass of antibodies, according to the following method.

### Genetic Construction of Mutant IL-15/Fc--

10 cDNA for Fcγ2a can be generated from mRNA extracted from an IgG2a secreting hybridoma using standard techniques with reverse transcriptase (MMLV-RT; Gibco-BRL, Grand Island, NY) and a synthetic oligo-dT (12-18) oligonucleotide (Gibco BRL). The mutant IL-15  
15 cDNA can be amplified from a plasmid template by PCR using IL-15 specific synthetic oligonucleotides.

The 5' oligonucleotide is designed to insert a unique NotI restriction site 40 nucleotides 5' to the translational start codon, while the 3' oligonucleotide eliminates the termination codon and modifies the C-terminal Ser residue codon usage from AGC to TCG to accommodate the creation of a unique BamHI site at the mutant IL-15/Fc junction. Synthetic oligonucleotides used for the amplification of the Fcγ2a domain cDNA  
25 change the first codon of the hinge from Glu to Asp in order to create a unique BamHI site spanning the first codon of the hinge and introduce a unique XbaI site 3' to the termination codon. The Fc fragment can be modified so that it is non-lytic, i.e., not able to  
30 activate the complement system. To make the non-lytic mutant IL-15 construct (mIL-15/Fc--), oligonucleotide site-directed mutagenesis is used to replace the C'1q binding motif Glu318, Lys320, Lys322 with Ala residues. Similarly, Leu235 is replaced with Glu to inactivate the  
35 FcγR I binding site. Ligation of cytokine and Fc"

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Proliferation of PHA-Stimulated Human PBMCs in the Presence of IL-15 Related Proteins

Human PBMCs prestimulated with PHA (2  $\mu$ g/ml) for 72 hours were washed and cultured in the presence of IL-15 related proteins including: (1) the IL-15 double mutant, FLAG-HMK-IL-15-Q149D-Q156D, (2) the IL-15 single mutant, FLAG-HMK-IL-15-Q149D, or (3) the IL-15 single mutant, FLAG-HMK-IL-15-Q156D (Fig. 16). Medium without an IL-15 related polypeptide served as a control. The proliferative response was then assessed.

FACS Analysis of PHA-Activated Human PBMCs Stained with FLAG-HMK-IL-15-Double Mutants

The ability of the FLAG-HMK-IL-15 double mutant polypeptide to bind PHA-activated human PBMCs was demonstrated as follows. PHA-activated PBMCs were washed and incubated with medium alone, or with the FLAG-HMK-IL-15 double mutant. The cells were then incubated with an anti-FLAG biotinylated antibody and stained with streptavidin conjugated to RED670. The stained cells were analyzed by flow cytometry (Fig. 17).

FACS Analysis of Leukemic Cell Lines Stained with Wild-Type FLAG-HMK-IL-15

In a series of experiments similar to those above, the ability of the wild-type FLAG-HMK-IL-15 polypeptide to bind leukemia cells was shown. The cells treated were obtained from the leukemic cell lines MOLT-14, YT, HuT-102, and from cell lines currently being established at Beth Israel Hospital (Boston, MA), and named 2A and 2B. The cultured cells were washed and incubated with either medium alone or with medium containing the FLAG-HMK-IL-15 wild-type polypeptide (Fig. 18). The cells were then incubated with the biotinylated anti-FLAG antibody and stained with RED670-conjugated streptavidin. The stained cells were analyzed by flow cytometry.

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Western blot analysis following SDS-PAGE under reducing (with DTT) and non-reducing (without DTT) conditions can be performed using monoclonal or polyclonal anti-mIL-15 or anti Fcγ primary antibodies to evaluate the size and isotype specificity of the fusion proteins.

Standardization of the Biological Activity of recombinant mutant IL-15 and mIL-15/Fc-- proteins

- Using the RT-PCR strategy and 5' NotI sense oligonucleotide primer described above, mutant IL-15 cDNA with an XbaI restriction site added 3' to its native termination codon, can be cloned into pRc/CMV. This construct is then transiently expressed in COS cells (available from the American Type Culture Collection).
- 15 The cells may be transfected by the DEAE dextran method and grown in serum-free UltraCulture media (BioWhittaker Inc.). Day 5 culture supernatant is sterile filtered and stored at -20°C for use as a source of recombinant mutant IL-15 protein (rmIL-15).
- 20 Mutant IL-15/Fc-- and rmIL-15 mutant protein concentrations can be determined by ELISA as well as by bioassay as described (Thompson-Snipes et al., J. Exp. Med. 173:507 (1991)).

The functional activity of mutant IL-15/Fc-- can be assessed by a standard T cell proliferation assay, as described above.

Determination of mIL-15/Fc-- or mIL-15/Fc++ Circulating Half-life

- Serum concentration of mIL-15/Fc or mIL-15/Fc++ fusion proteins can be determined over time following a single intravenous injection of the fusion protein. Serial 100 μl blood samples can be obtained by standard measures at intervals of 0.1, 6, 24, 48, 72, and 96 hours after administration of mutant IL-15/Fc-- protein.
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components in the correct translational reading frame at the unique *Bam*HI site yields a 1,236 basepair open reading frame encoding a single 411 amino acid polypeptide (including the 18 amino acid IL-15 signal peptide) with a total of 13 cysteine residues. The mature secreted homodimeric IL-15/Fc-- is predicted to have a total of up to eight intramolecular and three inter-heavy chain disulfide linkages and a molecular weight of approximately 85 kDa, exclusive of 10<sup>5</sup> glycosylation.

#### Expression and Purification of mIL-15 Fc Fusion Proteins

Proper genetic construction of both mIL-15/Fc++, which carries the wild-type Fcγ2a sequence, and mIL-15/Fc-- can be confirmed by DNA sequence analysis following cloning of the fusion genes as *Not*I-*Xba*I cassettes into the eukaryotic expression plasmid pRc/CMV (Invitrogen, San Diego, CA). This plasmid carries a CMV promoter/enhancer, a bovine growth hormone polyadenylation signal and a neomycin resistance gene for selection with G418. Plasmids carrying the mIL-15/Fc++ or mIL-15/Fc-- fusion genes are transfected into Chinese hamster ovary cells (CHO-K1, available from the American Type Culture Collection) by electroporation (1.5 kV/3 μF/0.4 cm/PBS) and selected in serum-free Ultra-CHO media (BiWhittaker Inc., Walkerville, MD) containing 1.5 mg/ml of G418 (Geneticin, Gibco BRL). After subcloning, clones that produce high levels of the fusion protein are selected by screening supernatants from IL-15 by ELISA (PharMingen, San Diego, CA). mIL-15/Fc fusion proteins are purified from culture supernatants by protein A sepharose affinity chromatography followed by dialysis against PBS and 0.22 μm filter sterilization. Purified proteins can be stored at -20°C before use.

- 54 -

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

5

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CGGGATCCTC AAGAAGTGTT GATGAA 26

## (2) INFORMATION FOR SEQ ID NO:3:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 60 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

10

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

15 CGGGATCCTC AAGAAGTGTT GATGAACATG TCGACAATAT GTACAAACT GTCCAAAAT 60

## (2) INFORMATION FOR SEQ ID NO:4:

## (i) SEQUENCE CHARACTERISTICS:

20

- (A) LENGTH: 7 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Asp Tyr Lys Asp Asp Asp Lys  
 1 5

25

## (2) INFORMATION FOR SEQ ID NO:5:

## (i) SEQUENCE CHARACTERISTICS:

30

- (A) LENGTH: 489 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

## (ix) FEATURE:

- (A) NAME/KEY: Coding Sequence  
 (B) LOCATION: 1...486  
 (D) OTHER INFORMATION:

35

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

ATG AGA ATT TCG AAA CCA CAT TTG AGA AGT ATT TCC ATC CAG TGC TAC 48  
 Met Arg Ile Ser Lys Pro His Leu Arg Ser Ile Ser Ile Gln Cys Tyr  
 1 5 10 15

40

TTG TGT TTA CTT CTA AAC AGT CAT TTT CTA ACT GAA GCT GGC ATT CAT 96  
 Leu Cys Leu Leu Leu Asn Ser His Phe Leu Thr Glu Ala Gly Ile His  
 20 25 30

GTC TTC ATT TTG GGC TGT TTC AGT GCA GGG CTT CCT AAA ACA GAA GCC 144  
 Val Phe Ile Leu Gly Cys Phe Ser Ala Gly Leu Pro Lys Thr Glu Ala  
 35 40 45

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Measurements employ an ELISA with a mIL-15 mAb as the capture antibody and horseradish peroxidase conjugated to an Fc<sup>2</sup>a mAb as the detection antibody, thus assuring this assay is specific for only the mutant IL-15/Fc--.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION

- (i) APPLICANT: BETH-ISRAEL HOSPITAL ASSOCIATION  
(ii) TITLE OF THE INVENTION: ANTAGONISTS OF INTERLEUKIN-15  
(iii) NUMBER OF SEQUENCES: 7

## (iv) CORRESPONDENCE ADDRESS:

- (A) ADDRESSEE: Fish & Richardson, P.C.  
(B) STREET: 225 Franklin Street  
(C) CITY: Boston  
(D) STATE: MA  
(E) COUNTRY: US  
(F) ZIP: 02110-2804

## (v) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Diskette  
(B) COMPUTER: IBM Compatible  
(C) OPERATING SYSTEM: Windows95  
(D) SOFTWARE: FastSEQ for Windows Version 2.0

## (vi) CURRENT APPLICATION DATA:

- (A) APPLICATION NUMBER: PCT/US97/06931  
(B) FILING DATE: 25-APR-1997  
(C) CLASSIFICATION: C12N 01/21

## (vii) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBER: 60/016,634  
(B) FILING DATE: 26-APR-1996

## (viii) ATTORNEY/AGENT INFORMATION:

- (A) NAME: Freeman, John W.  
(B) REGISTRATION NUMBER: 29,066  
(C) REFERENCE/DOCKET NUMBER: 01948/039W01

## (ix) TELECOMMUNICATION INFORMATION:

- (A) TELEPHONE: 617-542-5070  
(B) TELEFAX: 617-542-8906

## (2) INFORMATION FOR SEQ ID NO:1:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GGAATTCAAC TGGGTGAATG TAATA 25

## (2) INFORMATION FOR SEQ ID NO:2:

- 56 -

Val Ile Ser Leu Glu Ser Gly Asp Ala Ser Ile His Asp Thr Val Glu  
100 105 110

Asn Leu Ile Ile Leu Ala Asn Asn Ser Leu Ser Ser Asn Gly Asn Val  
115 120 125

5 Thr Glu Ser Gly Cys Lys Glu Cys Glu Glu Leu Glu Glu Lys Asn Ile  
130 135 140

Lys Glu Phe Leu Gln Ser Phe Val His Ile Val Gln Met Phe Ile Asn  
145 150 155 160

Thr Ser

10 (2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 489 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

15

(ix) FEATURE:

- (A) NAME/KEY: Coding Sequence  
(B) LOCATION: 1..486  
(D) OTHER INFORMATION:

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

ATG AGA ATT TCG AAA CCA CAT TTG AGA AGT ATT TCC ATC CAG TGC TAC 48  
Met Arg Ile Ser Lys Pro His Leu Arg Ser Ile Ser Ile Gln Cys Tyr  
1 5 10 15

TTG TGT TTA CTT CTA AAC AGT CAT TTT CTA ACT GAA GCT GGC ATT CAT 96  
Leu Cys Leu Leu Leu Asn Ser His Phe Leu Thr Glu Ala Gly Ile His  
20 25 30

GTC TTC ATT TTG GGC TGT TTC AGT GCA GGG CTT CCT AAA ACA GAA GCC 144  
Val Phe Ile Leu Gly Cys Phe Ser Ala Gly Leu Pro Lys Thr Glu Ala  
35 40 45

30 AAC TGG GTG AAT GTA ATA AGT GAT TTG AAA AAA ATT GAA GAT CTT ATT 192  
Asn Trp Val Asn Val Ile Ser Asp Leu Lys Lys Ile Glu Asp Leu Ile  
50 55 60

CAA TCT ATG CAT ATT GAT GCT ACT TTA TAT ACG GAA AGT GAT GTT CAC 240  
Gln Ser Met His Ile Asp Ala Thr Leu Tyr Thr Glu Ser Asp Val His  
65 70 75 80

35 CCC AGT TGC AAA GTA ACA GCA ATG AAG TGC TTT CTC TTG GAG TTA CAA 288  
Pro Ser Cys Lys Val Thr Ala Met Lys Cys Phe Leu Leu Glu Leu Gln  
85 90 95

40 GTT ATT TCA CTT GAG TCC GGA GAT GCA AGT ATT CAT GAT ACA GTA GAA 336  
Val Ile Ser Leu Glu Ser Gly Asp Ala Ser Ile His Asp Thr Val Glu  
100 105 110

AAT CTG ATC ATC CTA GCA AAC AAC AGT TTG TCT TCT AAC GGG AAT GTA 384  
Asn Leu Ile Ile Leu Ala Asn Asn Ser Leu Ser Ser Asn Gly Asn Val  
115 120 125

45 ACA GAA TCT GGA TGC AAA GAA TGT GAG GAA CTG GAG GAA AAA AAT ATT 432  
Thr Glu Ser Gly Cys Lys Glu Cys Glu Glu Leu Glu Glu Lys Asn Ile

- 55 -

AAC TGG GTG AAT GTA ATA AGT GAT TTG AAA AAA ATT GAA GAT CTT ATT 192  
 Asn Trp Val Asn Val Ile Ser Asp Leu Lys Lys Ile Glu Asp Leu Ile  
 50 55 60

5 CAA TCT ATG CAT ATT GAT GCT ACT TTA TAT ACG GAA AGT GAT GTT CAC 240  
 Gln Ser Met His Ile Asp Ala Thr Leu Tyr Thr Glu Ser Asp Val His  
 65 70 75 80

CCC AGT TGC AAA GTA ACA GCA ATG AAG TGC TTT CTC TTG GAG TTA CAA 288  
 Pro Ser Cys Lys Val Thr Ala Met Lys Cys Phe Leu Leu Glu Leu Gln  
 85 90 95

10 GTT ATT TCA CTT GAG TCC GGA GAT GCA AGT ATT CAT GAT ACA GTA GAA 336  
 Val Ile Ser Leu Glu Ser Gly Asp Ala Ser Ile His Asp Thr Val Glu  
 100 105 110

15 AAT CTG ATC ATC CTA GCA AAC AAC AGT TTG TCT TCT AAC GGG AAT GTA 384  
 Asn Leu Ile Ile Leu Ala Asn Asn Ser Leu Ser Ser Asn Gly Asn Val  
 115 120 125

ACA GAA TCT GGA TGC AAA GAA TGT GAG GAA CTG GAG GAA AAA AAT ATT 432  
 Thr Glu Ser Gly Cys Lys Glu Cys Glu Glu Leu Glu Glu Lys Asn Ile  
 130 135 140

20 AAA GAA TTT TTG CAG AGT TTT GTA CAT ATT GTC CAA ATG TTC ATC AAC 480  
 Lys Glu Phe Leu Gln Ser Phe Val His Ile Val Gln Met Phe Ile Asn  
 145 150 155 160

ACT TCT TGA 489  
 Thr Ser

## (2) INFORMATION FOR SEQ ID NO:6:

25 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 162 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

30 (v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Met Arg Ile Ser Lys Pro His Leu Arg Ser Ile Ser Ile Gln Cys Tyr  
 1 5 10 15

Leu Cys Leu Leu Leu Asn Ser His Phe Leu Thr Glu Ala Gly Ile His  
 20 25 30

Val Phe Ile Leu Gly Cys Phe Ser Ala Gly Leu Pro Lys Thr Glu Ala  
 35 40 45

Asn Trp Val Asn Val Ile Ser Asp Leu Lys Lys Ile Glu Asp Leu Ile  
 50 55 60

40 Gln Ser Met His Ile Asp Ala Thr Leu Tyr Thr Glu Ser Asp Val His  
 65 70 75 80

Pro Ser Cys Lys Val Thr Ala Met Lys Cys Phe Leu Leu Glu Leu Gln  
 85 90 95



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1. A substantially pure mutant IL-15 polypeptide, the polypeptide being:
  - (a) at least 90% identical to wild-type IL-15, and
  - (b) capable of inhibiting at least one of the5 cellular events that normally occurs when wild-type IL-15 specifically binds to a cell surface receptor.
2. The mutant polypeptide of claim 1, wherein the mutant polypeptide competes with wild-type IL-15 for binding to a cell surface receptor.
- 10 3. The mutant polypeptide of claim 1, wherein the mutant polypeptide binds to a cell surface receptor with at least as high an affinity as wild-type IL-15 binds the same cell surface receptor.
- 15 4. The mutant polypeptide of claim 1, wherein the mutant polypeptide binds the cell surface receptor with a  $K_d$  of greater than  $10^{-9}$  M.
5. The mutant polypeptide of claim 1, wherein at least one mutation is within the domain that binds IL-2R $\gamma$ .
- 20 6. The mutant polypeptide of claim 5, wherein the mutation is a deletion, insertion, or substitution of an amino acid residue.
7. The mutant polypeptide of claim 6, wherein the mutation is at position 156 of SEQ ID NO:6.
- 25 8. The mutant polypeptide of claim 7, further comprising a mutation at position 149 of SEQ ID NO:6.

- 57 -

130 135 140

AAA GAA TTT TTG GAC AGT TTT GTA CAT ATT GTC GAC ATG TTC ATC AAC 480  
 Lys Glu Phe Leu Asp Ser Phe Val His Ile Val Asp Met Phe Ile Asn  
 145 150 155 160

5 ACT TCT TGA 489  
 Thr Ser

## (2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 162 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

15 Met Arg Ile Ser Lys Pro His Leu Arg Ser Ile Ser Ile Gln Cys Tyr  
 1 5 10 15  
 Leu Cys Leu Leu Leu Asn Ser His Phe Leu Thr Glu Ala Gly Ile His  
 20 25 30  
 Val Phe Ile Leu Gly Cys Phe Ser Ala Gly Leu Pro Lys Thr Glu Ala  
 35 40 45  
 Asn Trp Val Asn Val Ile Ser Asp Leu Lys Lys Ile Glu Asp Leu Ile  
 50 55 60  
 Gln Ser Met His Ile Asp Ala Thr Leu Tyr Thr Glu Ser Asp Val His  
 65 70 75 80  
 25 Pro Ser Cys Lys Val Thr Ala Met Lys Cys Phe Leu Leu Glu Leu Gln  
 85 90 95  
 Val Ile Ser Leu Glu Ser Gly Asp Ala Ser Ile His Asp Thr Val Glu  
 100 105 110  
 Asn Leu Ile Ile Leu Ala Asn Asn Ser Leu Ser Ser Asn Gly Asn Val  
 115 120 125  
 Thr Glu Ser Gly Cys Lys Glu Cys Glu Glu Leu Glu Glu Lys Asn Ile  
 130 135 140  
 Lys Glu Phe Leu Asp Ser Phe Val His Ile Val Asp Met Phe Ile Asn  
 145 150 155 160  
 35 Thr Ser

What is claimed is:

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18. An isolated nucleic acid molecule encoding the polypeptide of claim 1.

19. A vector comprising the nucleic acid molecule of claim 18.

5 20. The vector of claim 19, wherein the vector is an expression vector.

21. A biological cell comprising the expression vector of claim 20.

22. A method of suppressing the immune response in a patient, the method comprising administering to the patient the mutant IL-15 polypeptide of claim 1.

23. The method of claim 22, wherein the patient has an autoimmune disease or is at risk of developing an autoimmune disease.

15 24. The method of claim 23, wherein the autoimmune disease is a rheumatic disease selected from the group consisting of systemic lupus erythematosus, Sjögren's syndrome, scleroderma, mixed connective tissue disease, dermatomyositis, polymyositis, Reiter's syndrome, and Behcet's disease.

20 25. The method of claim 23, wherein the autoimmune disease is rheumatic arthritis.

26. The method of claim 23, wherein the autoimmune disease is type I diabetes.

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9. The mutant polypeptide of claim 7, wherein the mutation at position 156 of SEQ ID NO:6 is a substitution of aspartate for glutamine.

10. The mutant polypeptide of claim 8, wherein the mutation at position 149 of SEQ ID NO:6 is a substitution of aspartate for glutamine.

11. The mutant polypeptide of claim 1, wherein the polypeptide is part of a chimeric molecule that comprises a heterologous polypeptide.

12. The mutant polypeptide of claim 11, wherein the heterologous polypeptide is the Fc region of IgG.

13. The polypeptide of claim 12, wherein the Fc region of IgG increases the circulating half-life of the mutant polypeptide.

14. The mutant polypeptide of claim 12, wherein the Fc region of IgG is capable of activating the complement lysis system and Fc receptor bearing phagocytes.

15. The mutant polypeptide of claim 12, wherein the Fc region of IgG is incapable of activating the complement lysis system.

16. The mutant polypeptide of claim 1, further comprising an antigenic tag.

17. The mutant polypeptide of claim 16, wherein the antigenic tag is a FLAG sequence comprising Asp-Tyr-Lys-Asp-Asp-Asp-Lys (SEQ ID NO:4).

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comprising exposing the cell to the mutant IL-15 polypeptide of claim 1.

36. The method of claim 35, wherein the cell is malignant.

5           37. A method of diagnosing a patient as having a disease or condition that could be treated with a mutant IL-15 polypeptide, the method comprising determining whether a biological sample obtained from the patient contains cells that are bound by a polypeptide comprising  
10 IL-15 and an antigenic tag, the occurrence of binding indicating that the cells can be bound by mutant IL-15 polypeptide *in vivo* and thereby inhibited from proliferating in response to wild-type IL-15 *in vivo*.



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27. The method of claim 23, wherein the autoimmune disease is an autoimmune disease of the thyroid selected from the group consisting of Hashimoto's thyroiditis and Graves' Disease.

5 28. The method of claim 23, wherein the autoimmune disease is an autoimmune disease of the central nervous system selected from the group consisting of multiple sclerosis, myasthenia gravis, and encephalomyelitis.

10 29. The method of claim 23, wherein the autoimmune disease is a variety of pemphigus selected from the group consisting of pemphigus vulgaris, pemphigus vegetans, pemphigus foliaceus, Senear-Usher syndrome, and Brazilian pemphigus.

15 30. The method of claim 23, wherein the autoimmune disease is psoriasis.

31. The method of claim 23, wherein the autoimmune disease is inflammatory bowel disease.

20 32. The method of claim 22, wherein the patient has acquired immune deficiency syndrome (AIDS).

33. The method of claim 22, wherein the patient has received a transplant of a biological organ, tissue, or cell.

25 34. The method of claim 22, wherein the patient has received a vascular injury.

35. A method of reducing the viability of a cell that expresses a receptor for IL-15, the method

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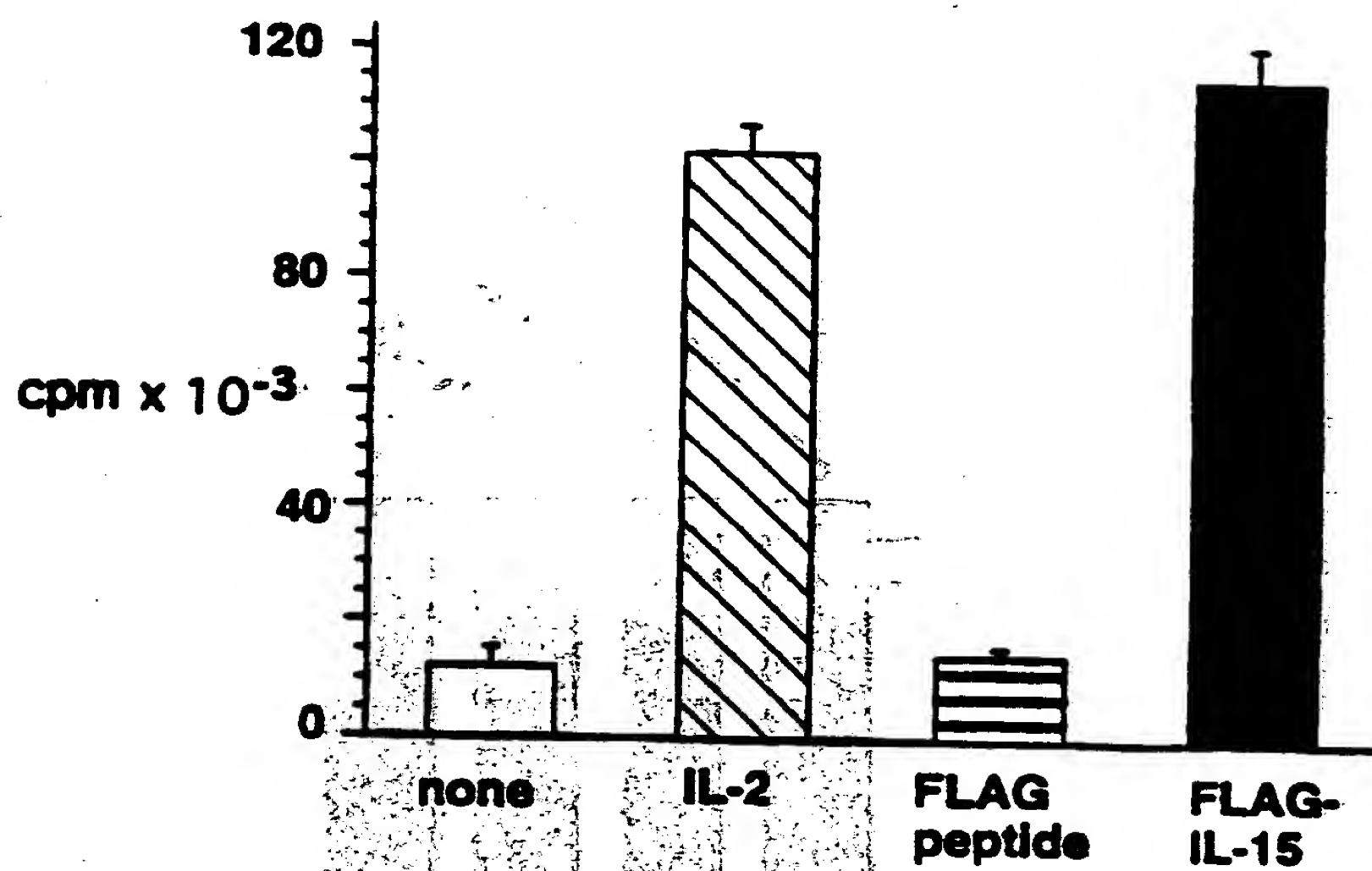


FIG. 3A

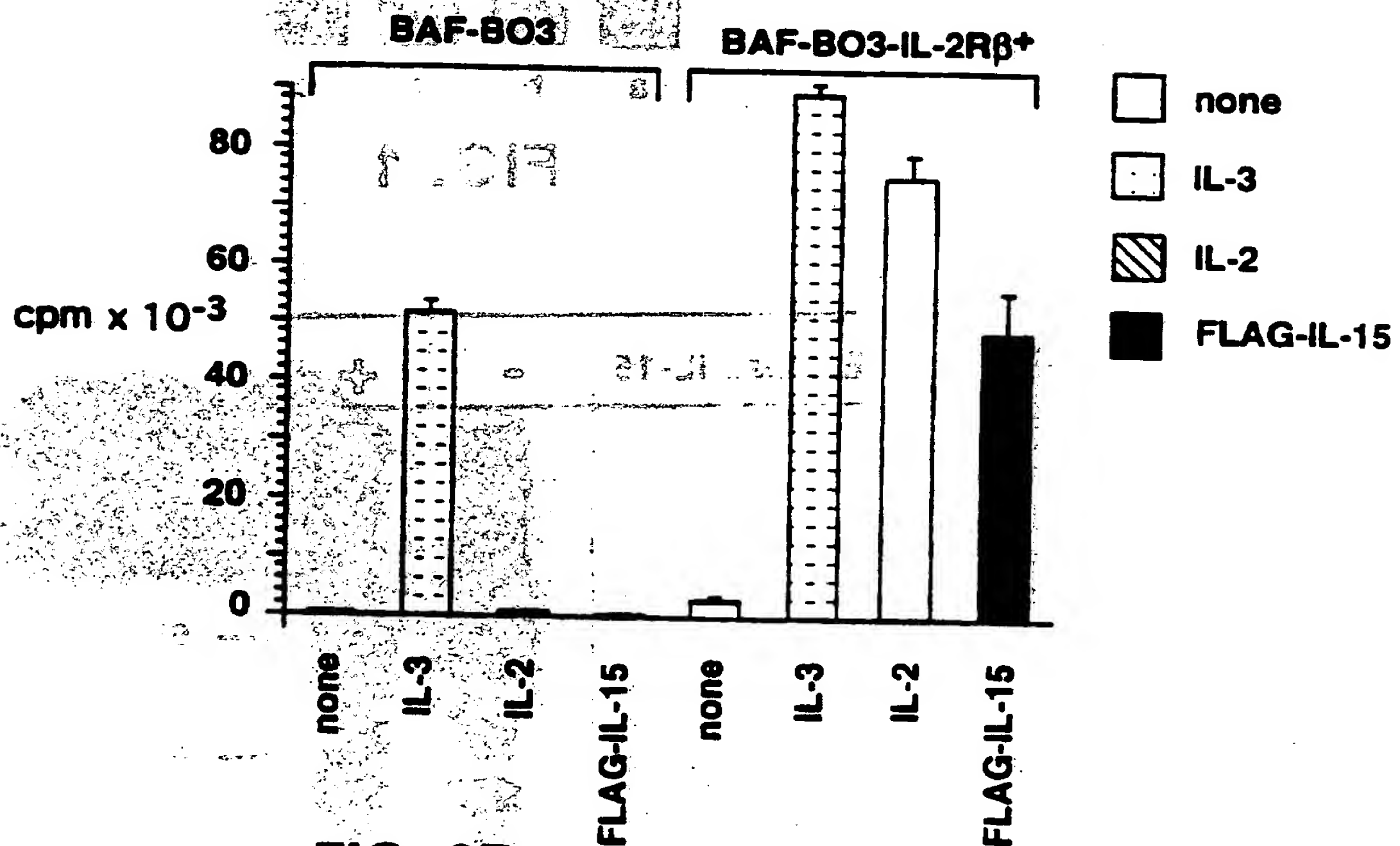


FIG. 3B

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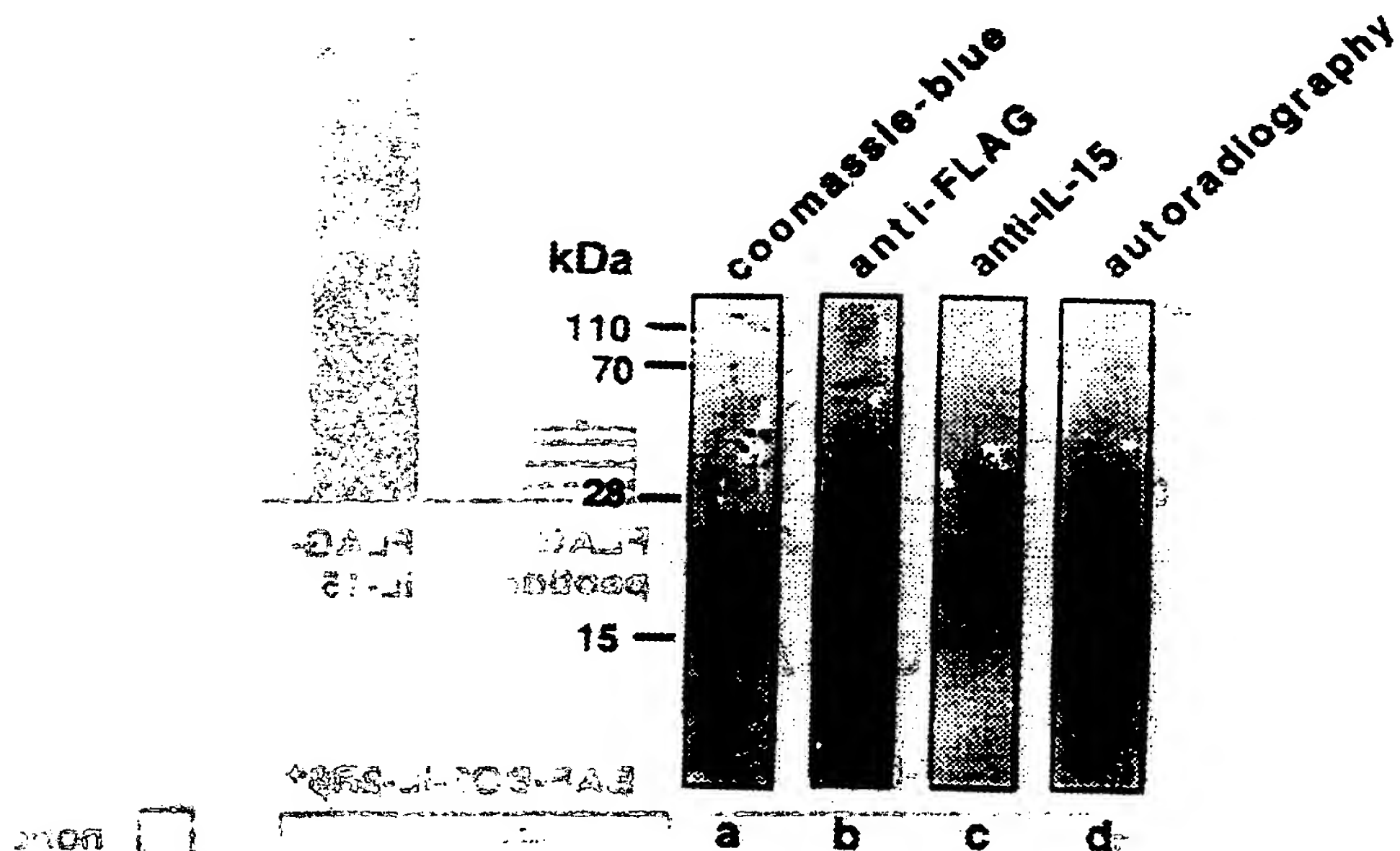


FIG. 1

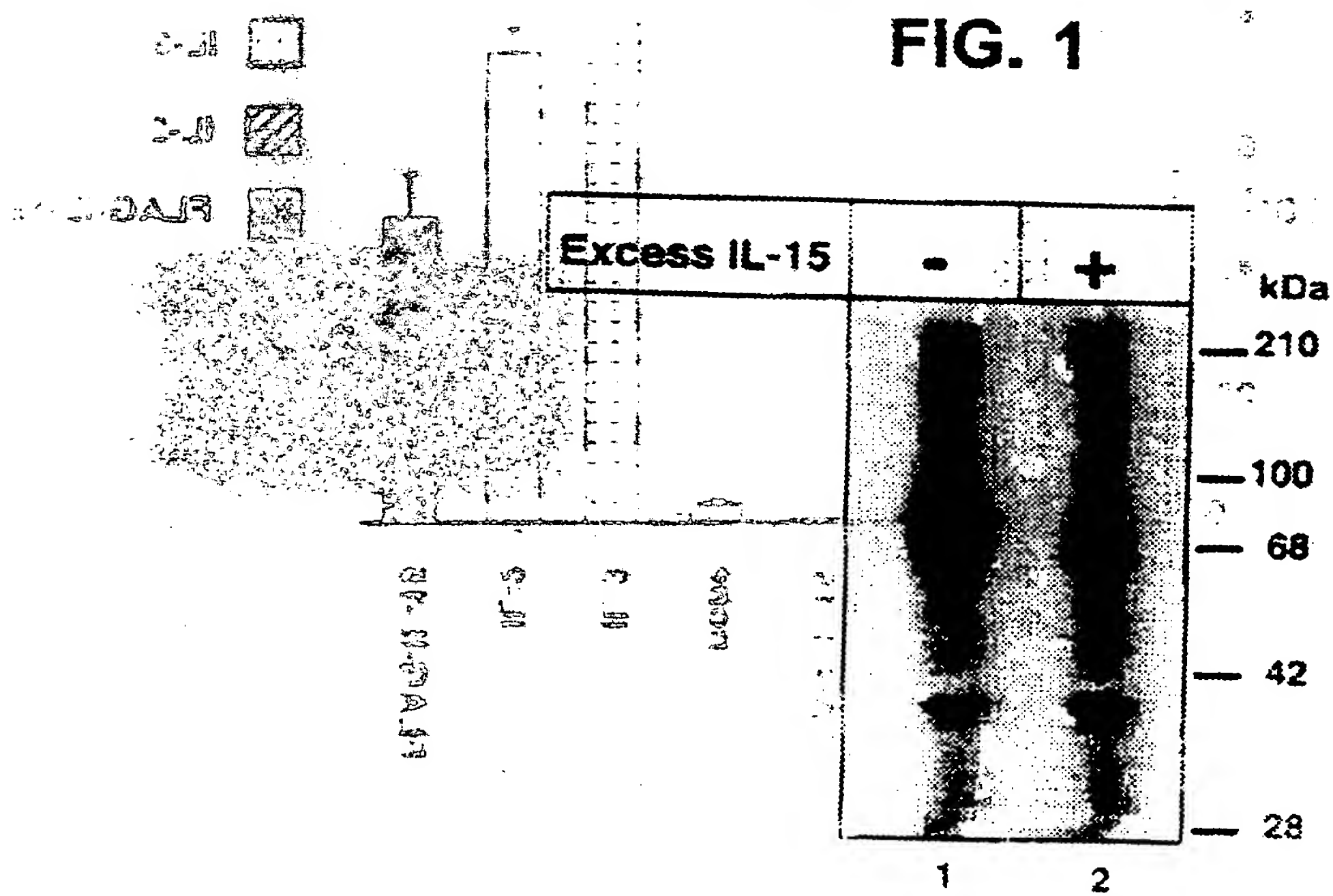


FIG. 2

SUBSTITUTE SHEET (RULE 26)

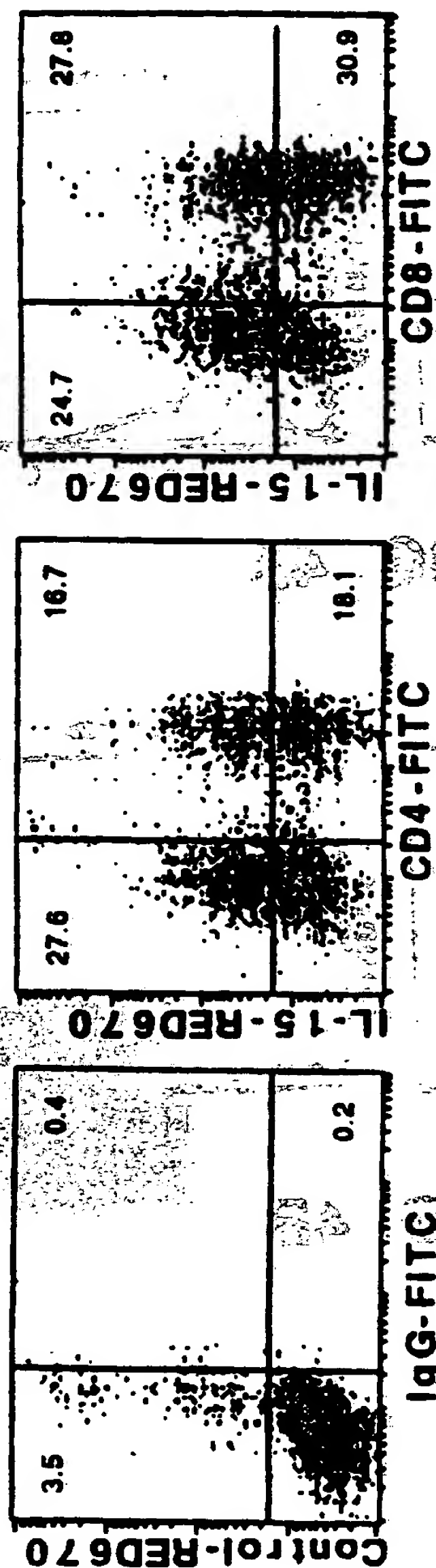


FIG. 4C

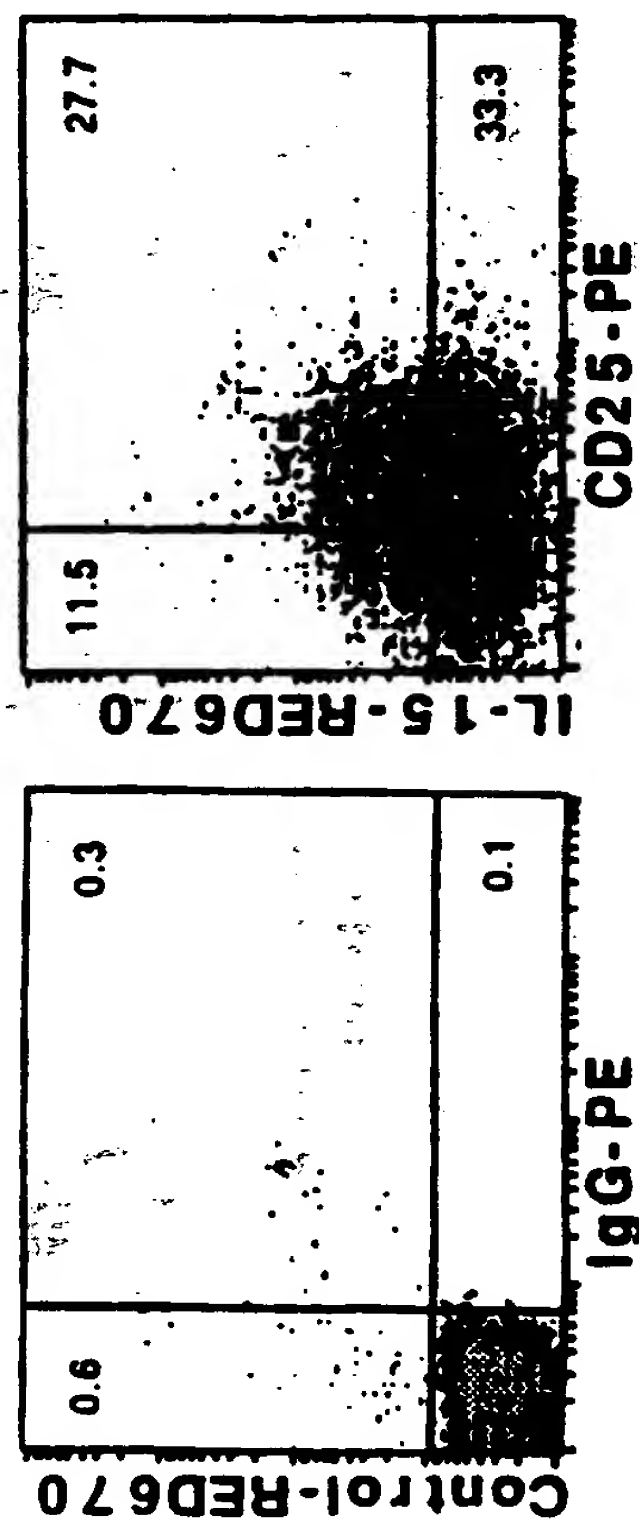
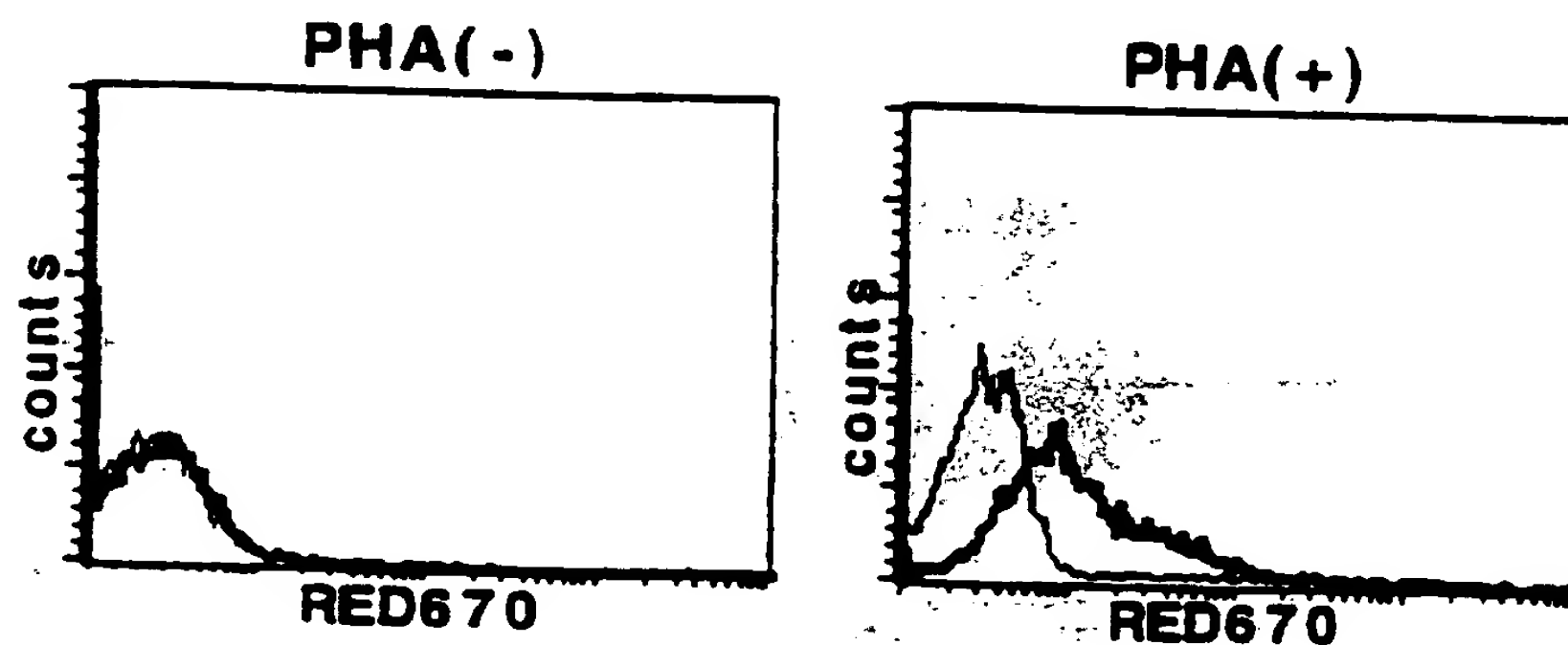
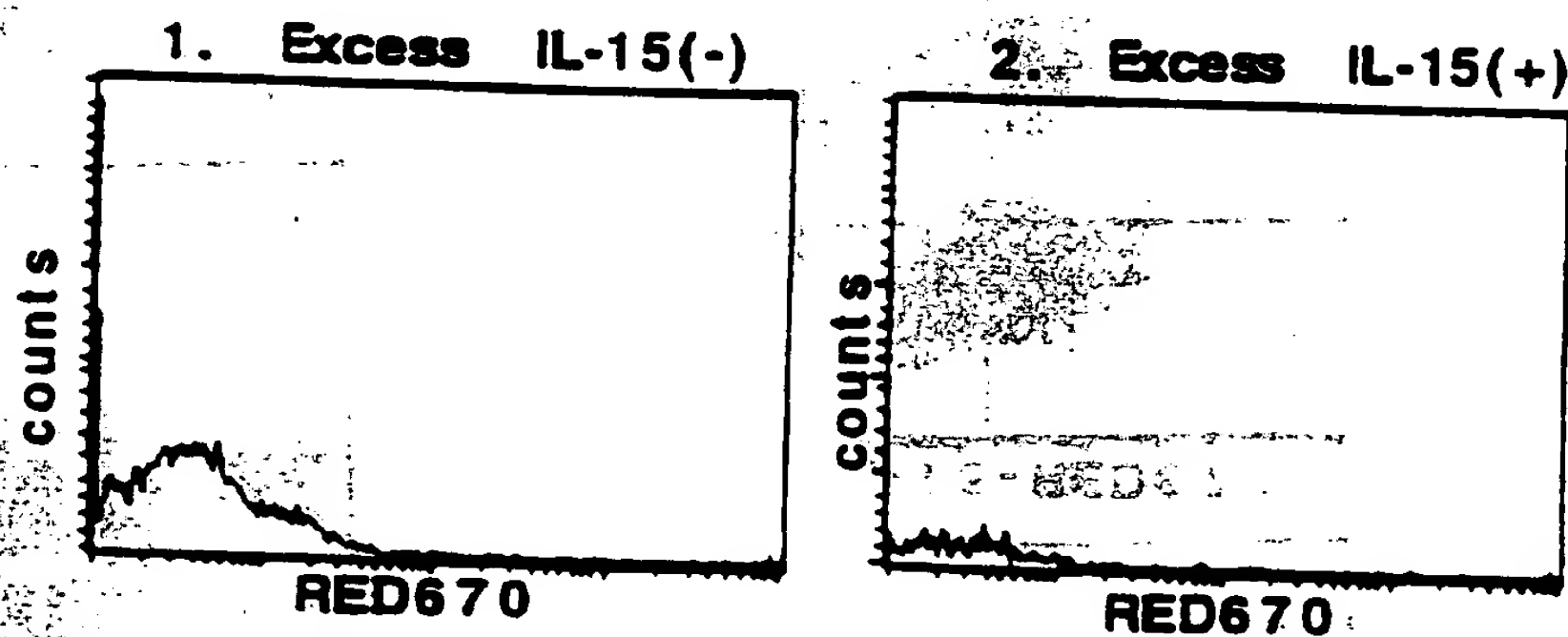


FIG. 5A

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**FIG. 4A****FIG. 4B**



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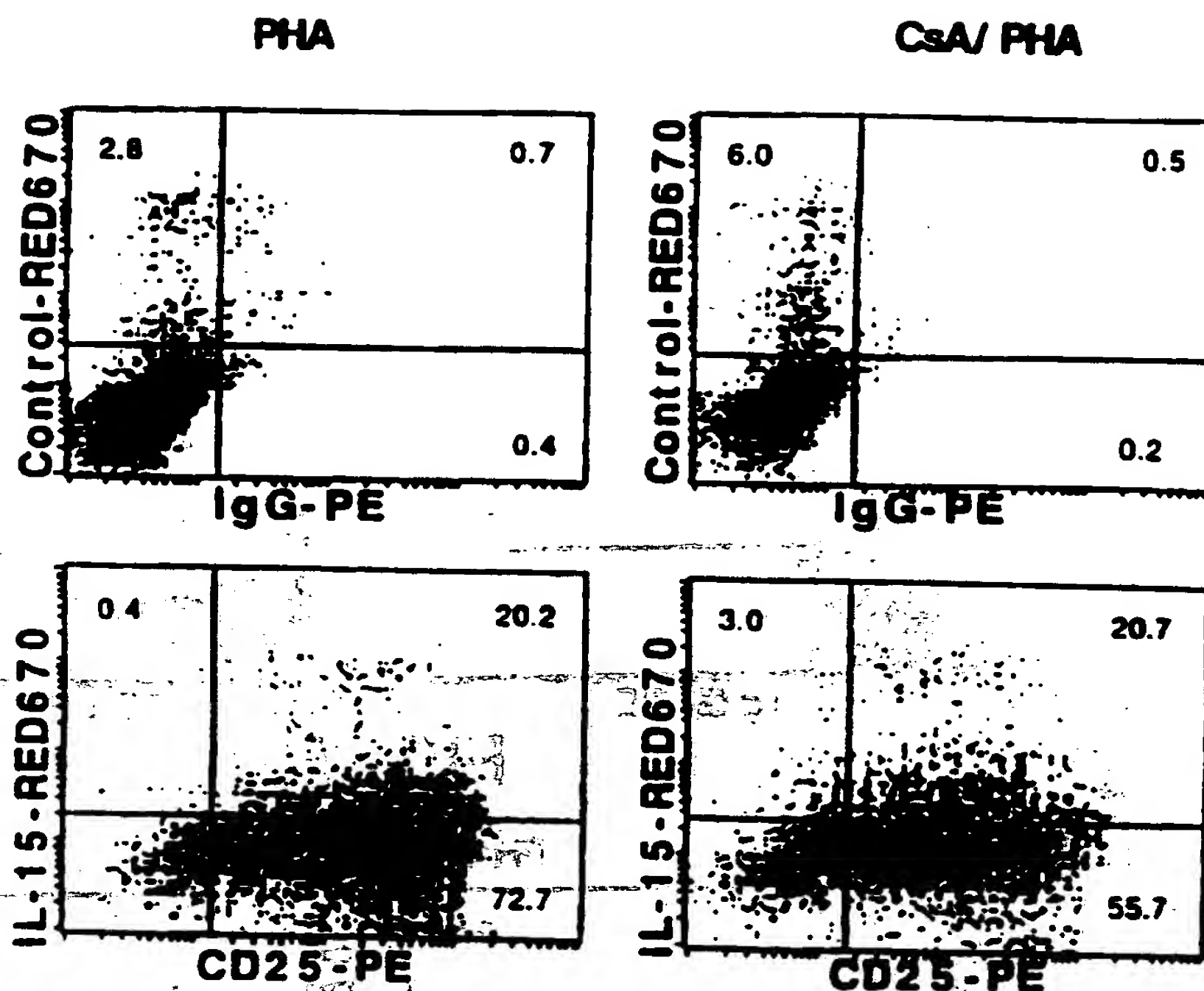


FIG. 6A

FIG. 6B

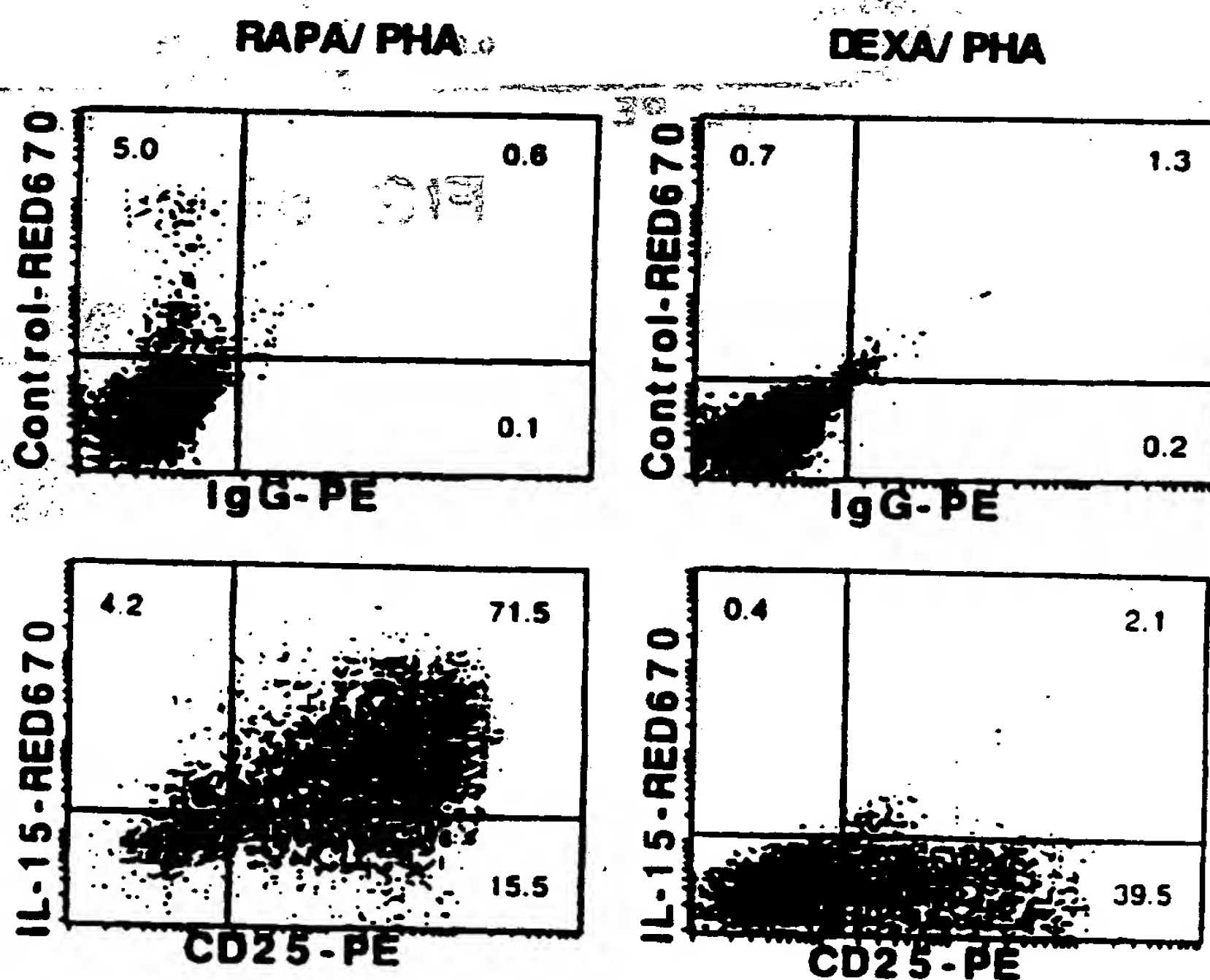


FIG. 6C

FIG. 6D

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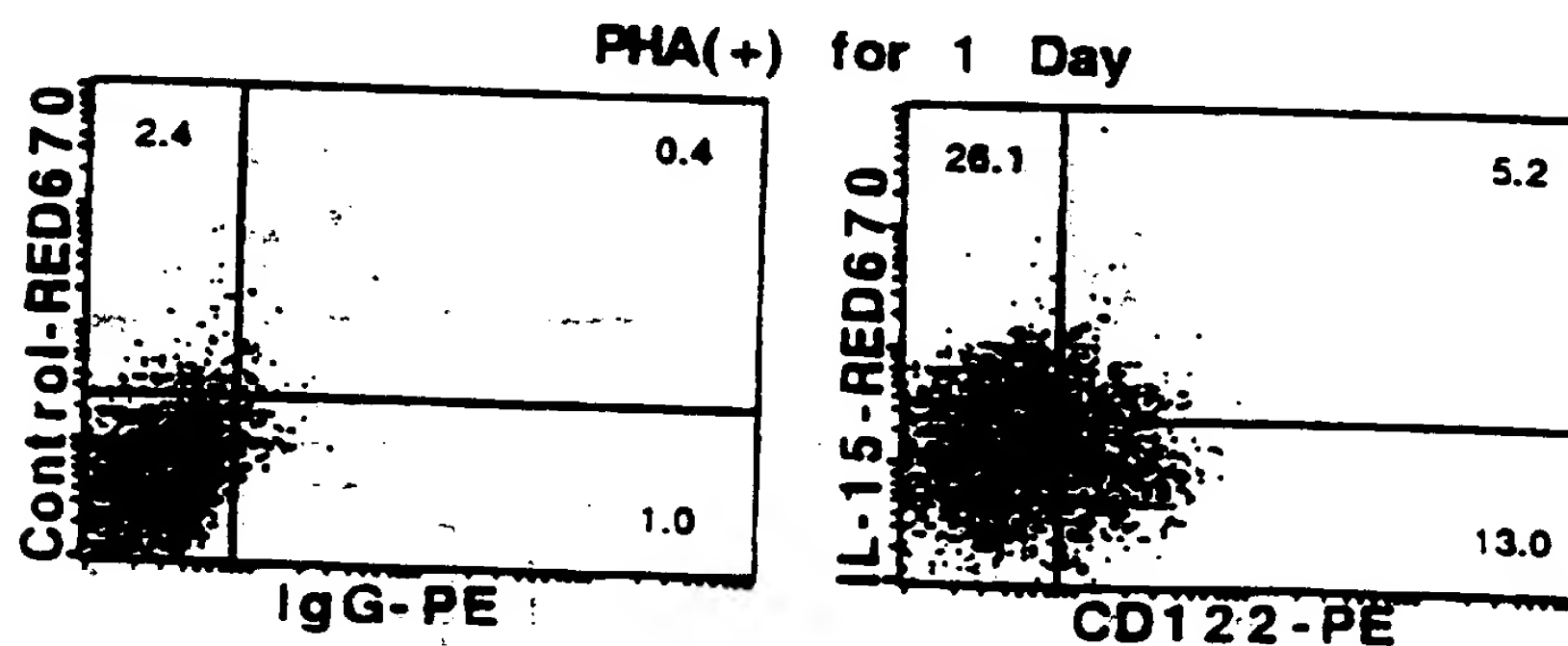


FIG. 5B

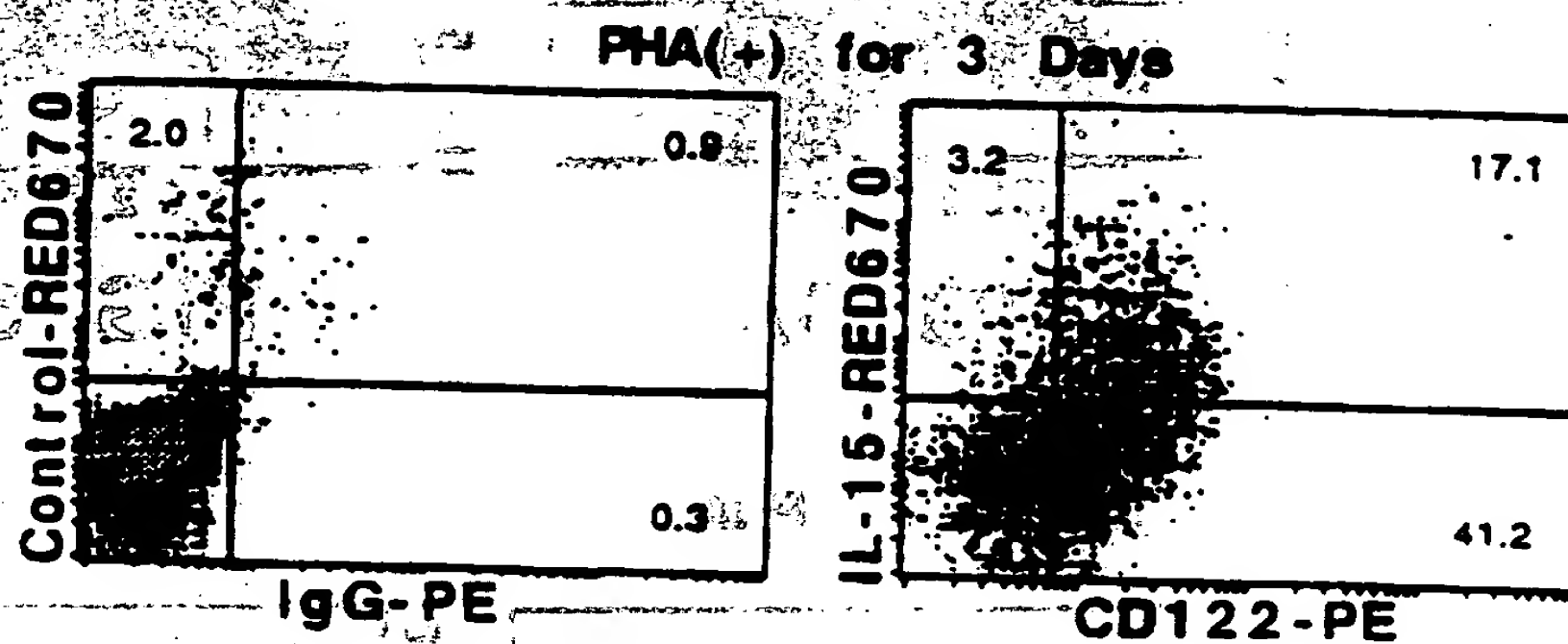


FIG. 5C

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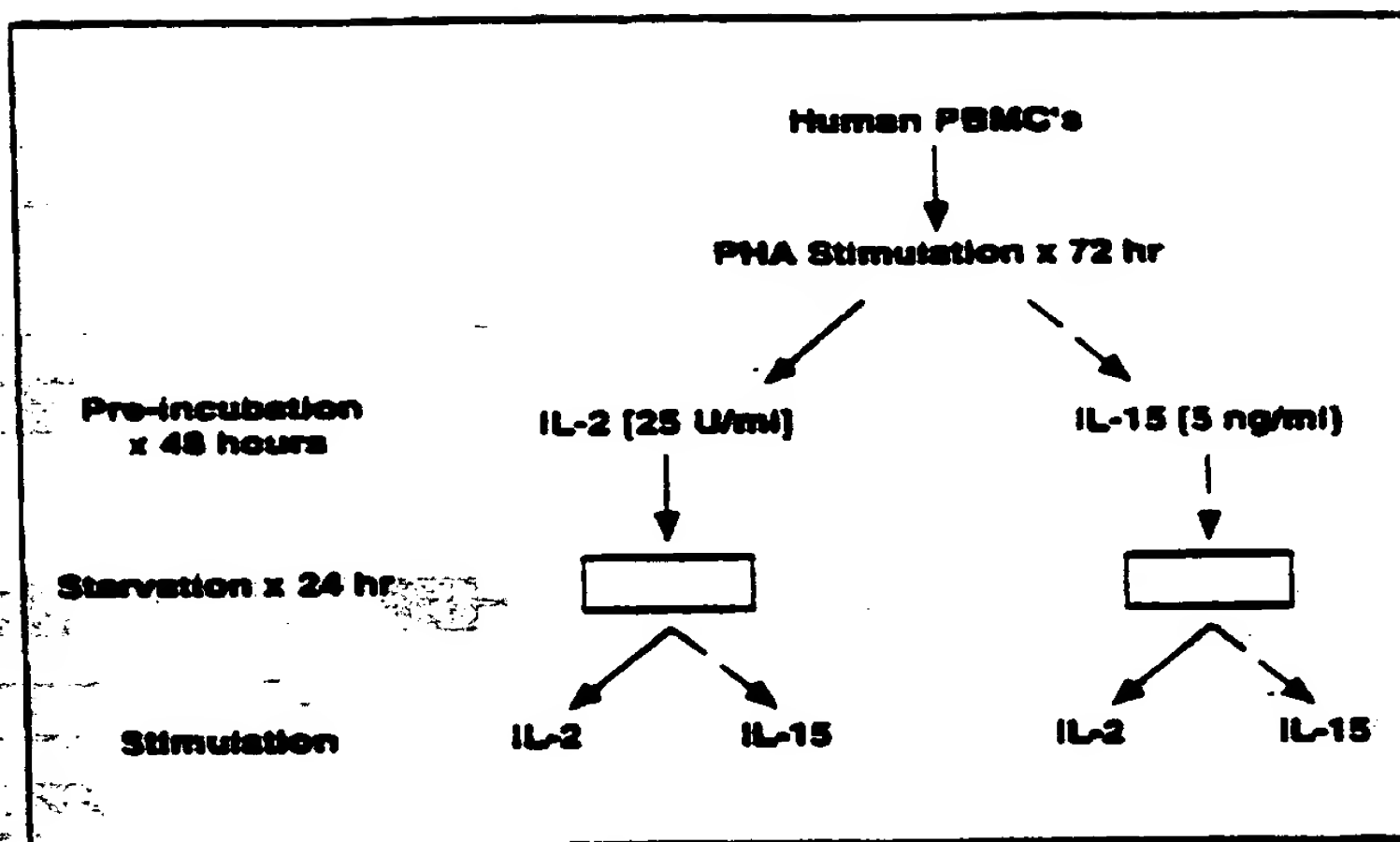


FIG. 8A

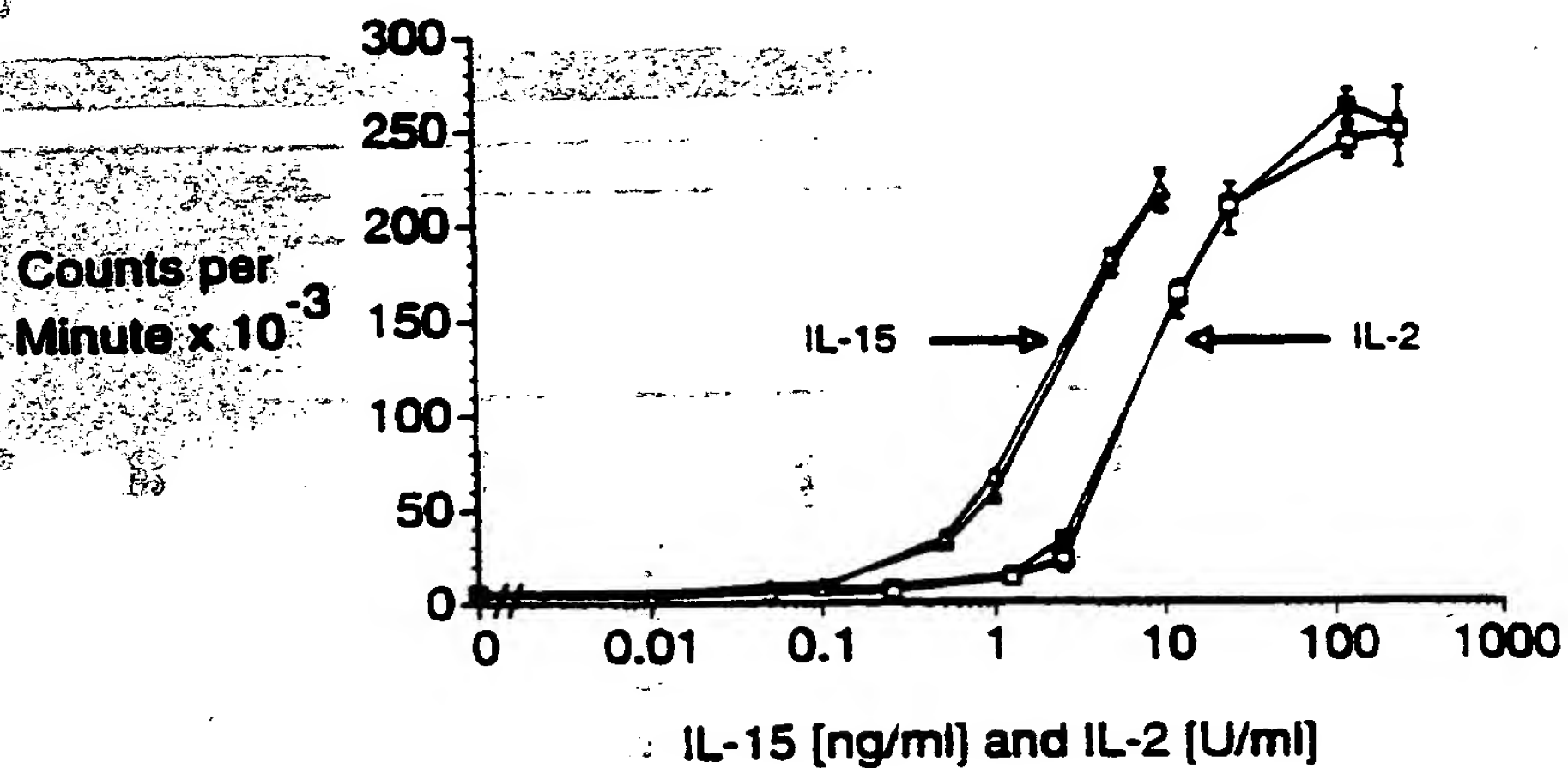


FIG. 8B

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Secondary culture  
in the presence of:

- none
- ▨ PHA
- ▤ IL-2
- IL-15

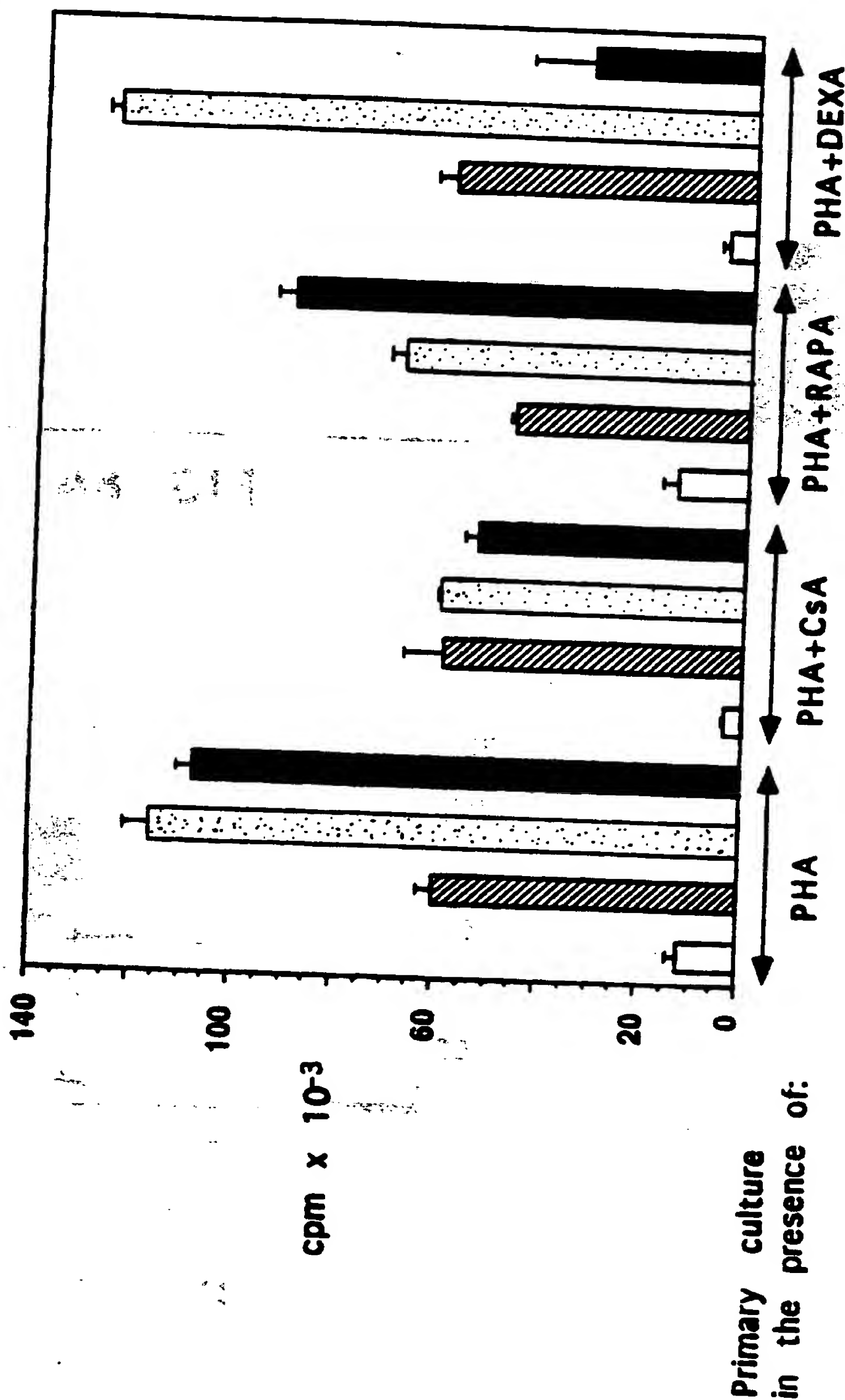


FIG. 7

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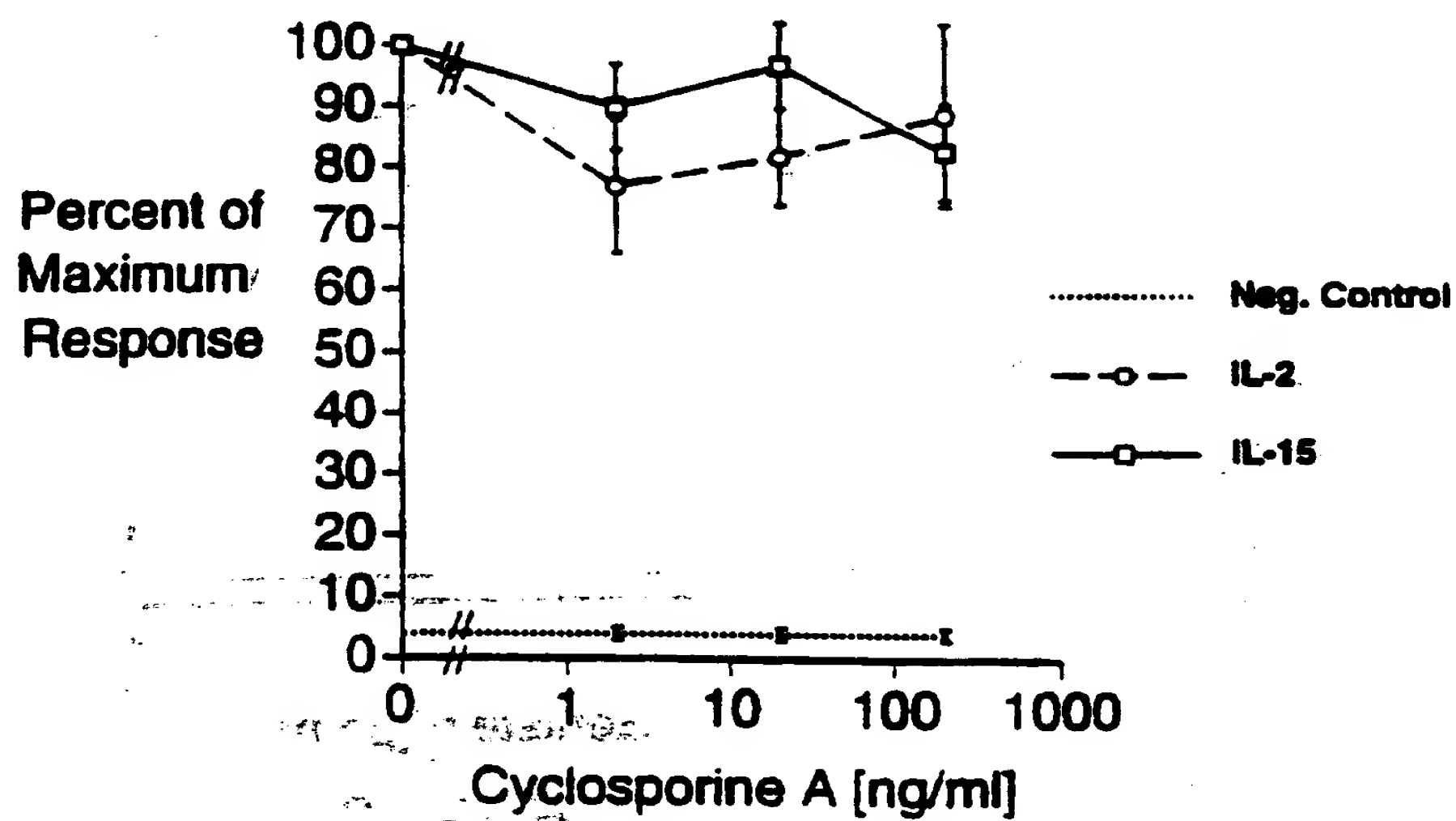


FIG. 11



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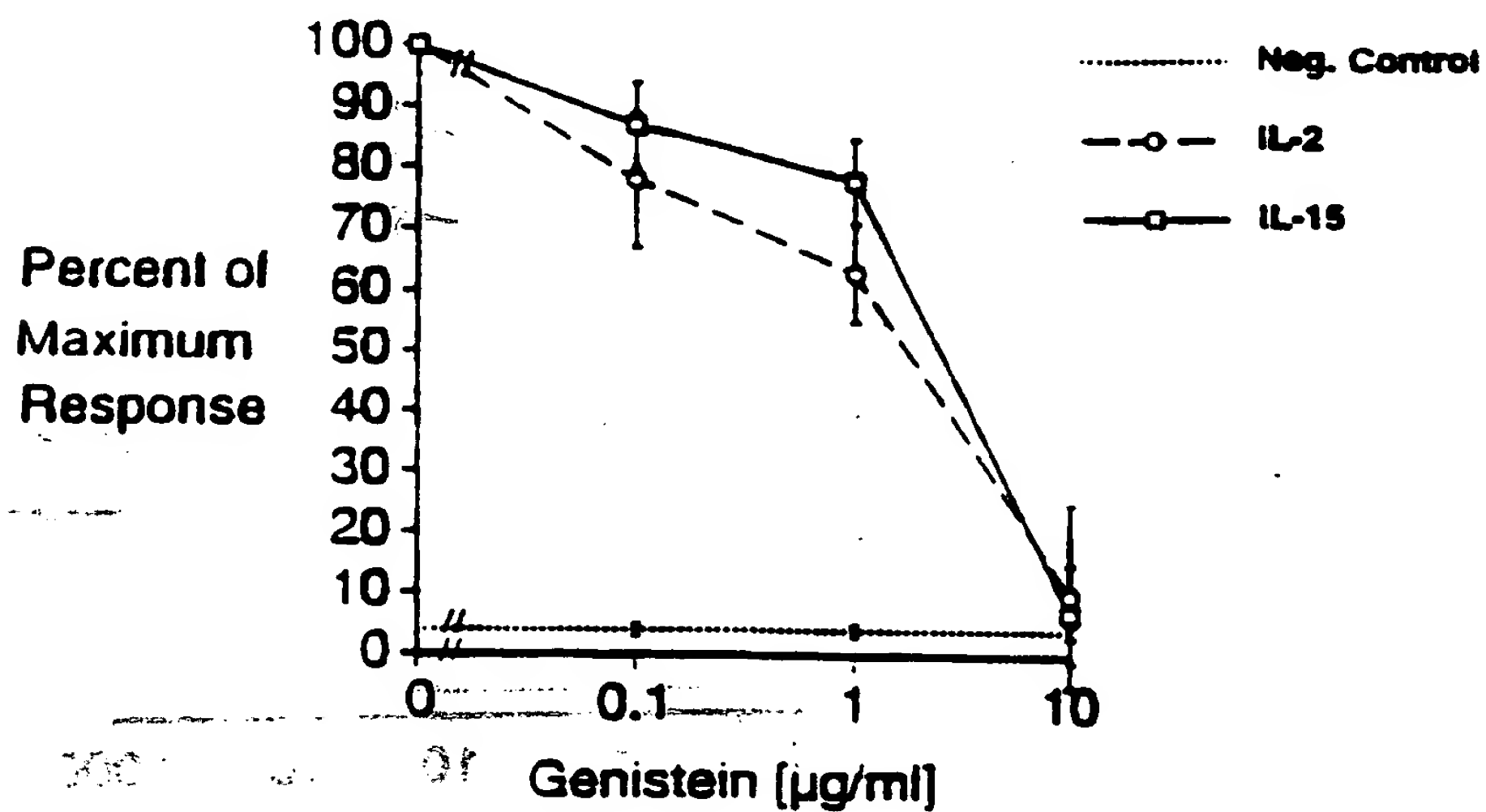


FIG. 9

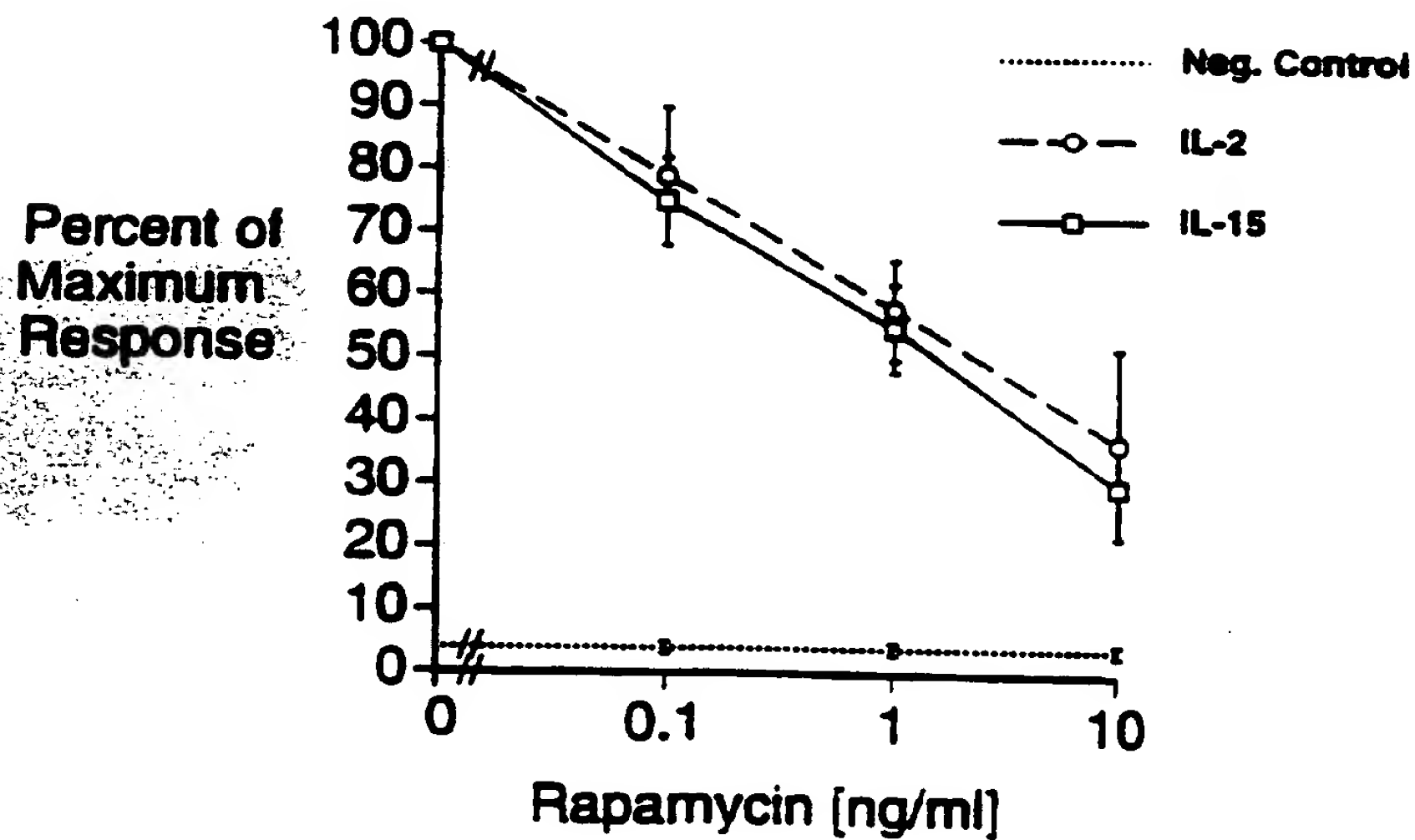


FIG. 10

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### DNA Strider™ 1.1 ### Friday, February 2, 1996 9:16:01

# hIL-15/Immunex/ORF -> 1-phase Translation

DNA sequence	489 b.p.	atgagaatttcg ... aacacttctga	linear
1/1	31/11		
atg aga att tcg aaa cca cat ttg aga agt att tcc atc cag tgc tac ttg tgt tta ctt			
Met arg ile ser lys pro his leu arg ser ile ser ile gln cys tyr leu cys leu leu			
61/21	91/31		
cta aac agt cat ttt cta act gaa gct ggc att cat gtc ttc att ttg ggc tgt ttc agt			
leu asn ser his phe leu thr glu ala gly ile his val phe ile leu gly cys phe ser			
121/41	151/51		
gca ggg ctt cct aaa aca gaa gcc aac tgg gtg aat gta ata agt gat ttg aaa aaa att			
ala gly leu pro lys thr glu ala asn trp val asn val ile ser asp leu lys lys ile			
181/61	211/71		
gaa gat ctt att caa tct atg cat att gat gct act tta tat acg gaa agt gat gtt cac			
glu asp leu ile gln ser met his ile asp ala thr leu tyr thr glu ser asp val his			
241/81	271/91		
ccc agt tgc aaa gta aca gca atg aag tgc ttt ctc ttg gag tta caa gtt att tca ctt			
pro ser cys lys val thr ala met lys cys phe leu leu glu leu gln val ile ser leu			
301/101	331/111		
gag tcc gga gat gca agt att cat gat aca gta gaa aat ctg atc atc cta gca aac aac			
glu ser gly asp ala ser ile his asp thr val glu asn leu ile ile leu ala asn asn			
361/121	391/131		
agt ttg tct tct aat ggg aat gta aca gaa tct gga tgc aaa gaa tgt gag gaa ctg gag			
ser leu ser ser asn gly asn val thr glu ser gly cys lys glu cys glu glu leu glu			
421/141	451/151		
gaa aaa aat att aaa gaa ttt ttg cag agt ttt gta cat att gtc caa atg ttc atc aac			
glu lys asn ile lys glu phe leu gln ser phe val his ile val gln met phe ile asn			
481/161			
act tct tga			
thr ser nda			

FIG. 13

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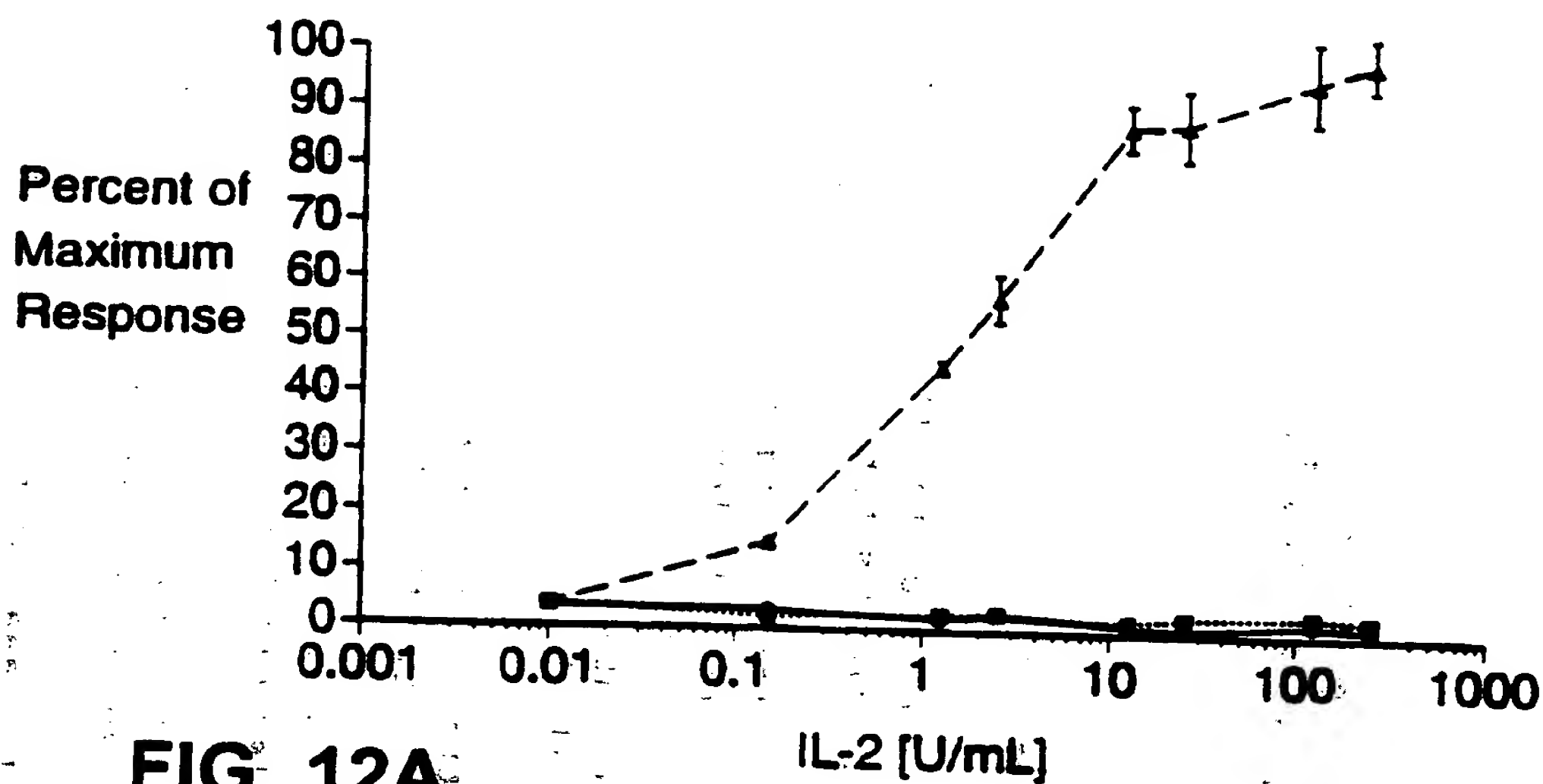


FIG. 12A

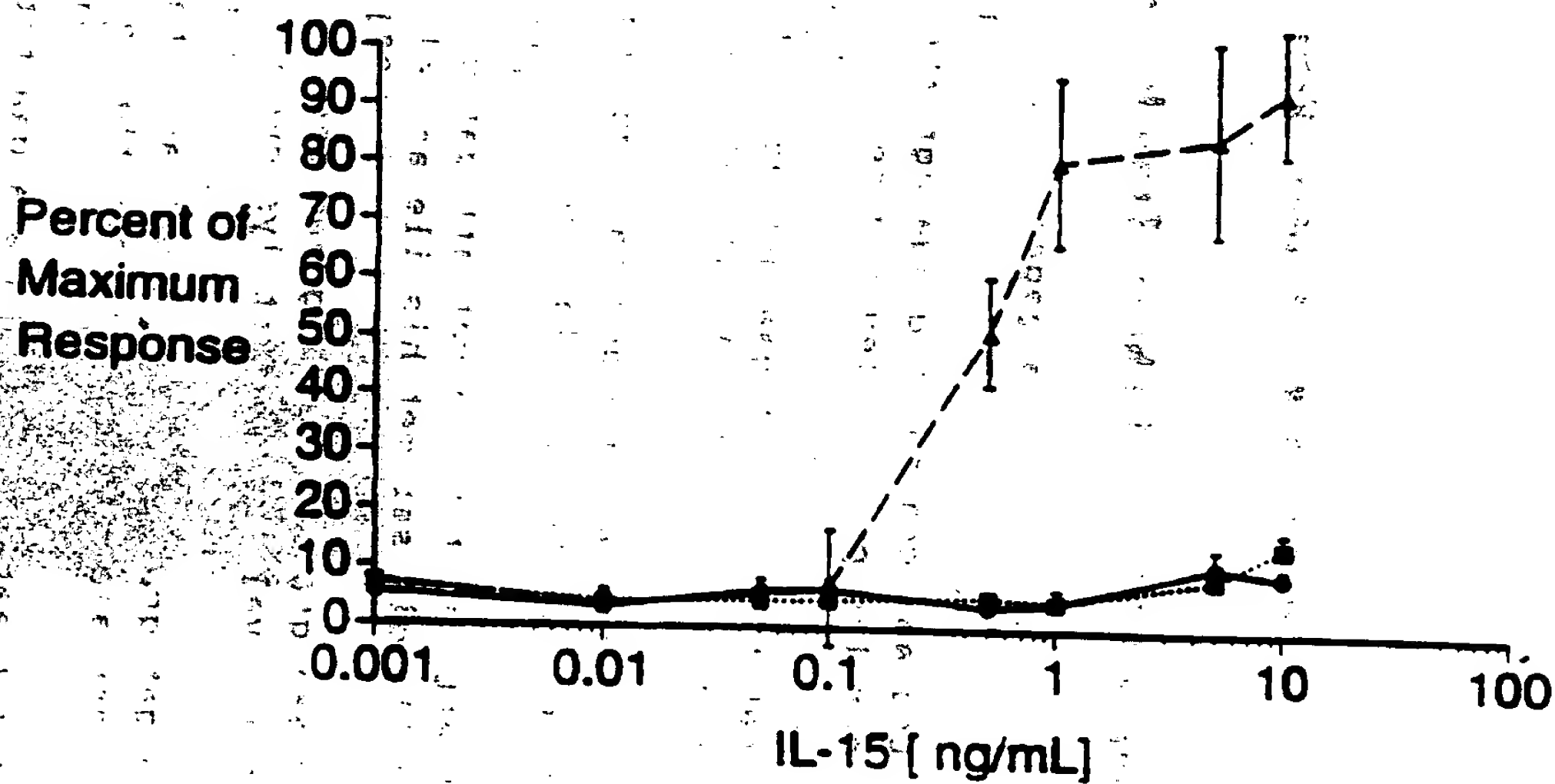
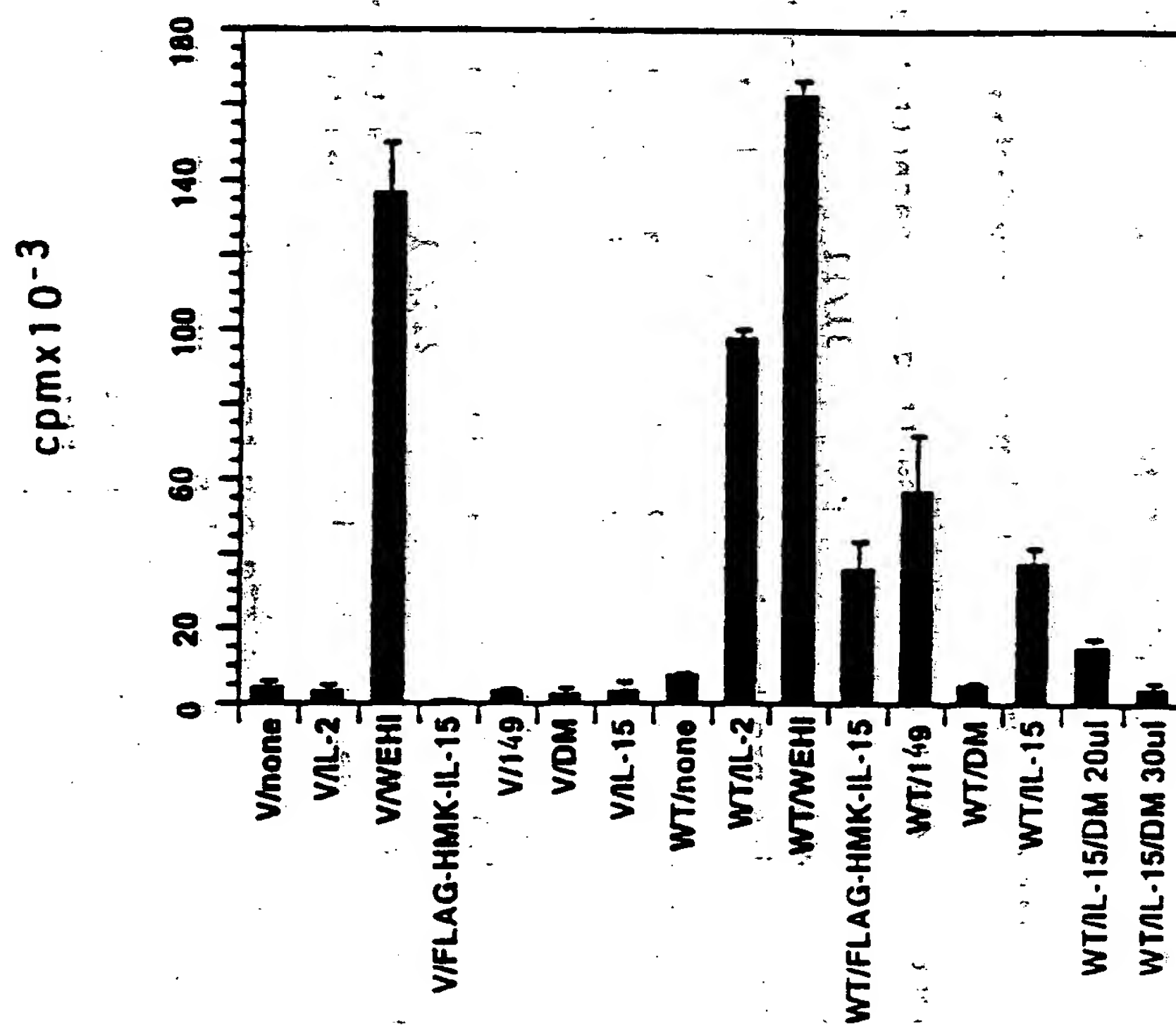


FIG. 12B

SUBSTITUTE SHEET (RULE 26)

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FIG. 15



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### DNA Strider™ 1.1 ### Friday, February 2, 1996 9:24:40

# human IL-15/169/176 mutant -> 1-phase Translation

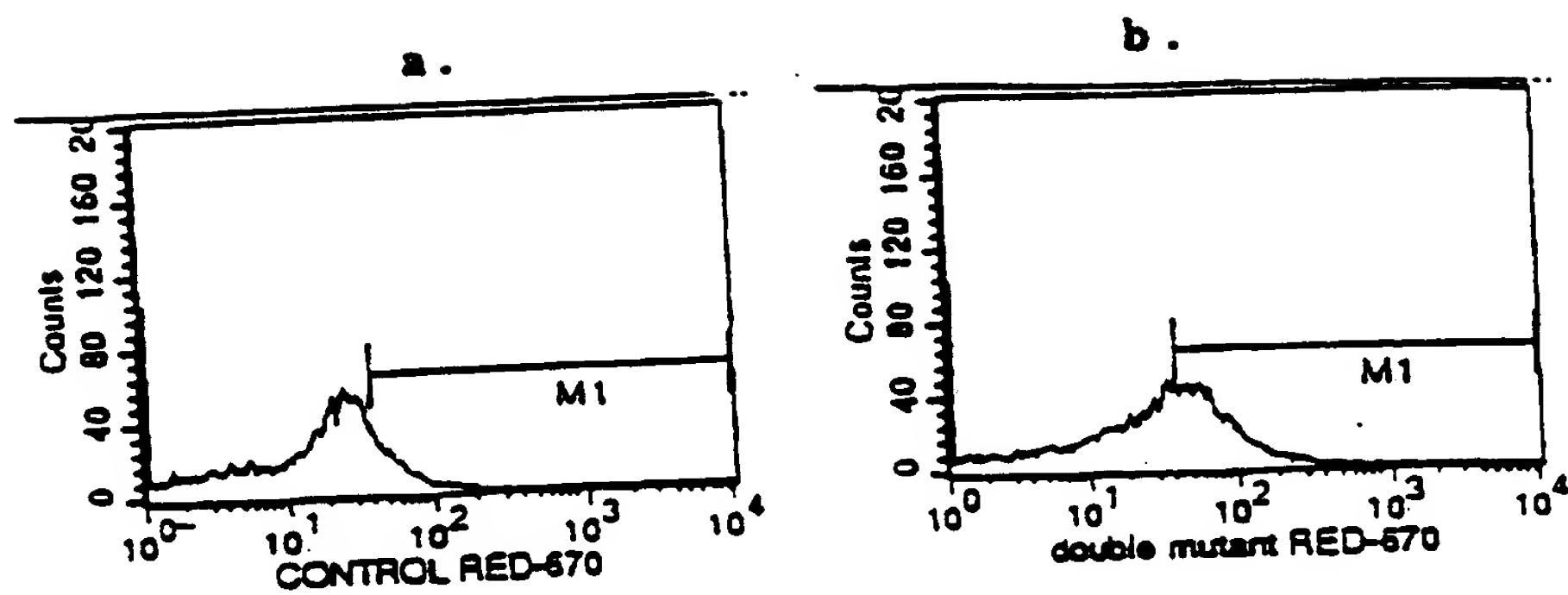
DNA sequence	489 b p	atgagaatttcg ... aacacttctga	linear
1/1			
atg aga att tcg aaaccca	31/11		tgc tac ttg tgt tta ctt
Met arg ile ser lys pro		cat ttg aga agt att tcc atc	cys tyr leu cys leu leu
61/21		his leu arg ser ile ser ile gln	
	91/31		
cta aac agt cat ttt cta act gaa gct ggc att cat gtc ttc			att ttg ggc tgt ttc agt
leu asn ser his phe leu thr glu ala gly ile his val phe			ile leu gly cys phe ser
121/41	151/51		
gca ggg ctt cct aaa aca gaa gcc aac tgg gtg aat gta ata			agt gat ttg aaa aaa att
ala gly leu pro lys thr glu ala asn trp val asn val ile			ser asp leu lys lys ile
181/61	211/71		
gaa gat ctt att caa tct atg cat att gat gct acc tta tat			acg gaa agt gat gtt cac
glu asp leu ile gln ser met his ile asp ala thr leu tyr			thr glu ser asp val his
241/81	271/91		
ccc agt tgc aaa gta aca gca atg aag tgc ttt etc ttg gag			tta caa gtt att tca ctt
pro ser cys lys val thr ala met lys cys phe leu leu glu			leu gln val ile ser leu
301/101	331/111		
gag tcc gga gat gca agt att cat gat aca gta gaa aat ctg atc atc cta gca aac aac			
glu ser gly asp ala ser ile his asp thr val glu asn leu ile ile leu ala asn asn			
361/121	391/131		
agt ttg tct tct aat ggg aat gta aca gaa tct gga tgc aaa gaa tgt gag gaa ctg gag			
ser leu ser ser asn gly asn val thr glu ser gly cys lys glu cys glu glu leu glu			
421/141	451/151		
gaa aaa aat att aaa gaa ttt ttg gac agt ttt gta cat att gtc gac atg ttc atc aac			
glu lys asn ile lys glu phe leu asp ser phe val his ile val asp met phe ile asn			
481/161			
act tct tga			
thr ser opa			

## FIG. 14



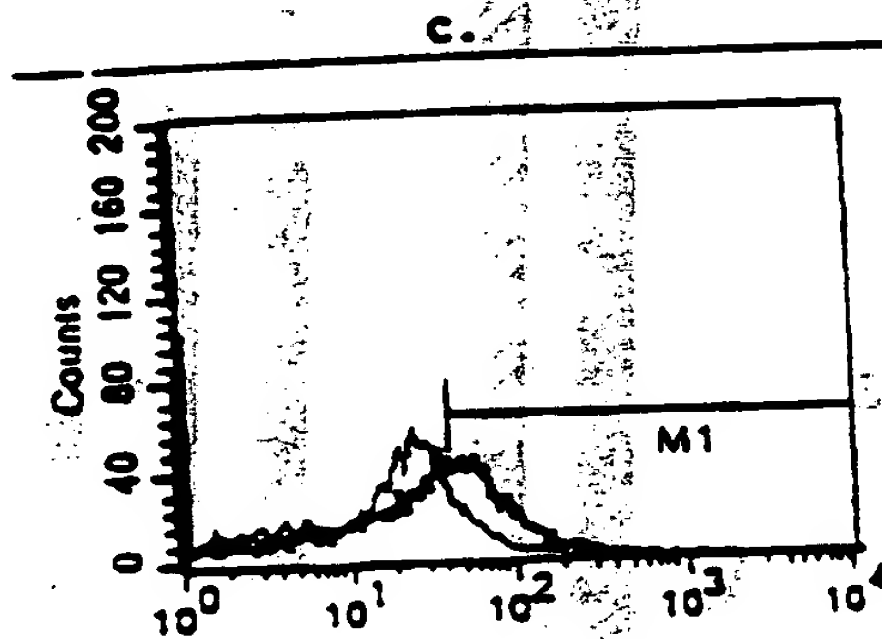
16/18

FIG. 17

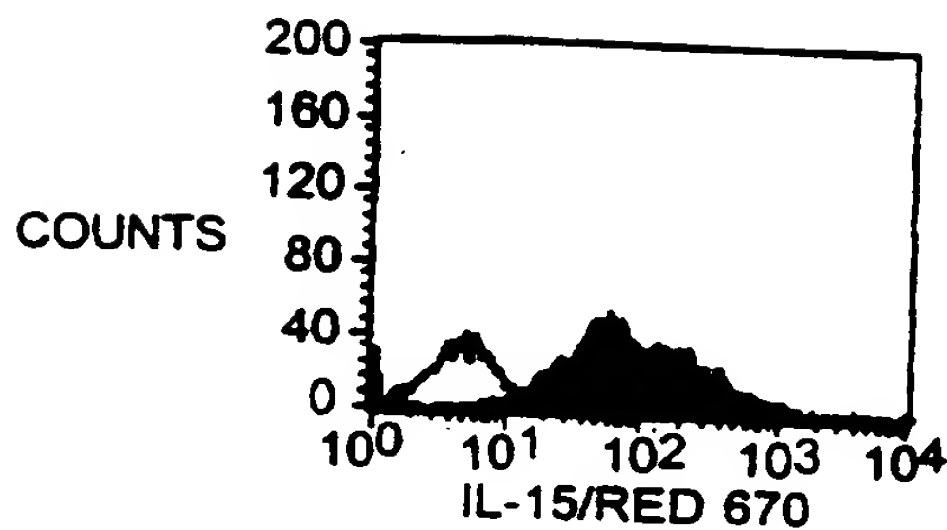


M1=8.8% of gated events

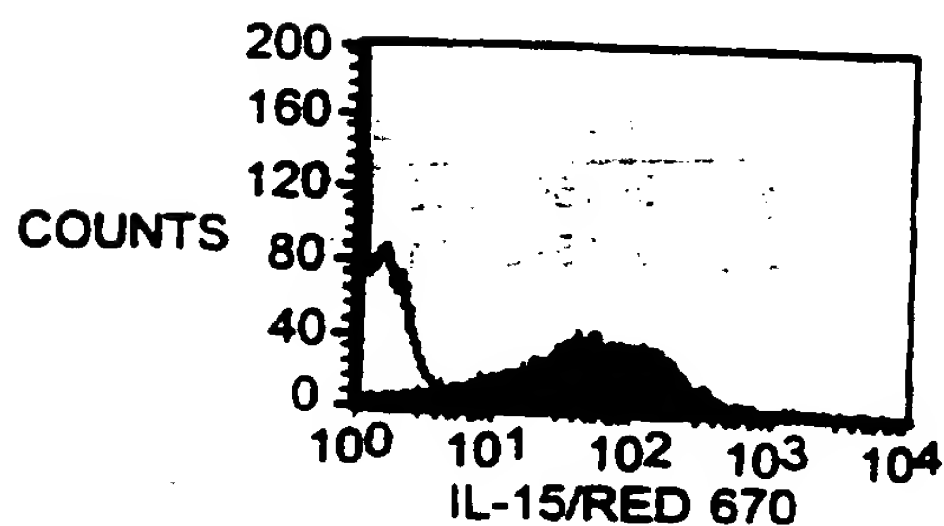
M1=32.6% of gated events



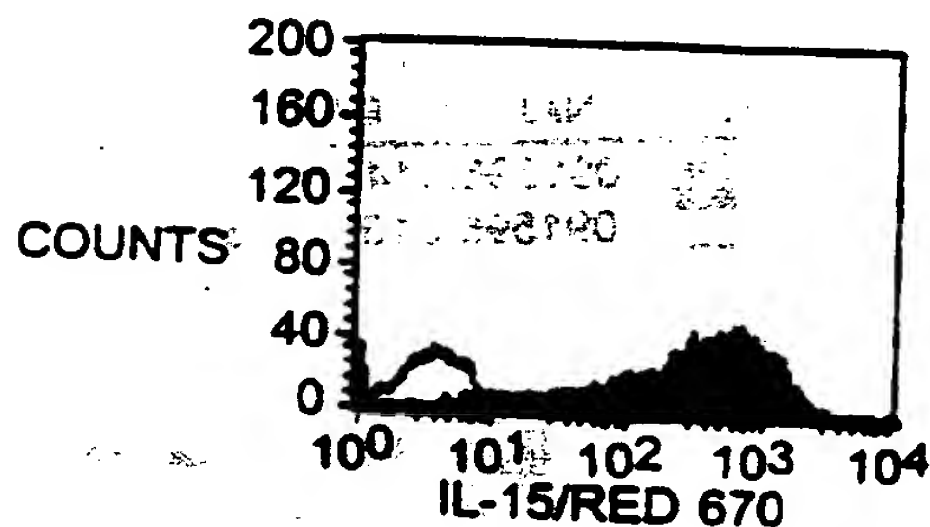
17/18



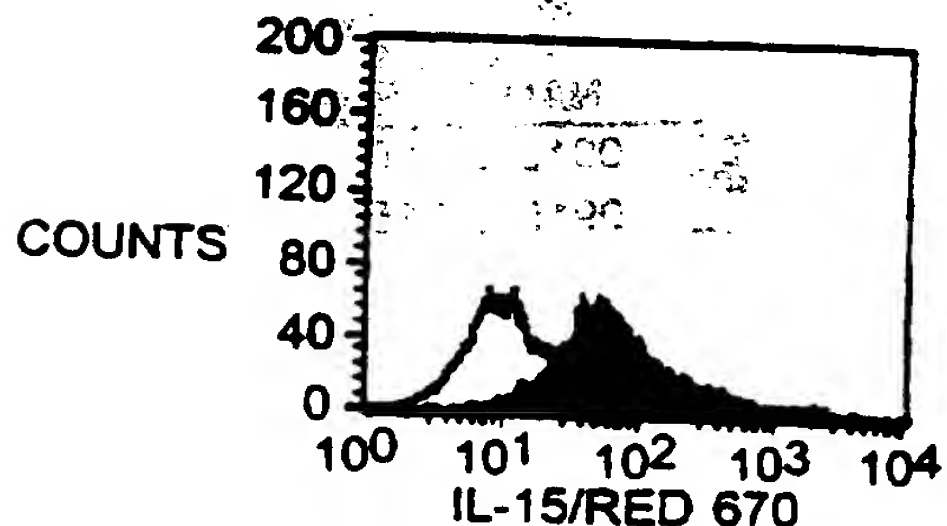
KEY	NAME	PARAMETER	GATE
■	091595.002	FL3-H	NO GATE POS
—	091595.001	FL3-H	NO GATE NEG



KEY	NAME	PARAMETER	GATE
■	091595.004	FL3-H	NO GATE POS
—	091595.003	FL3-H	NO GATE NEG



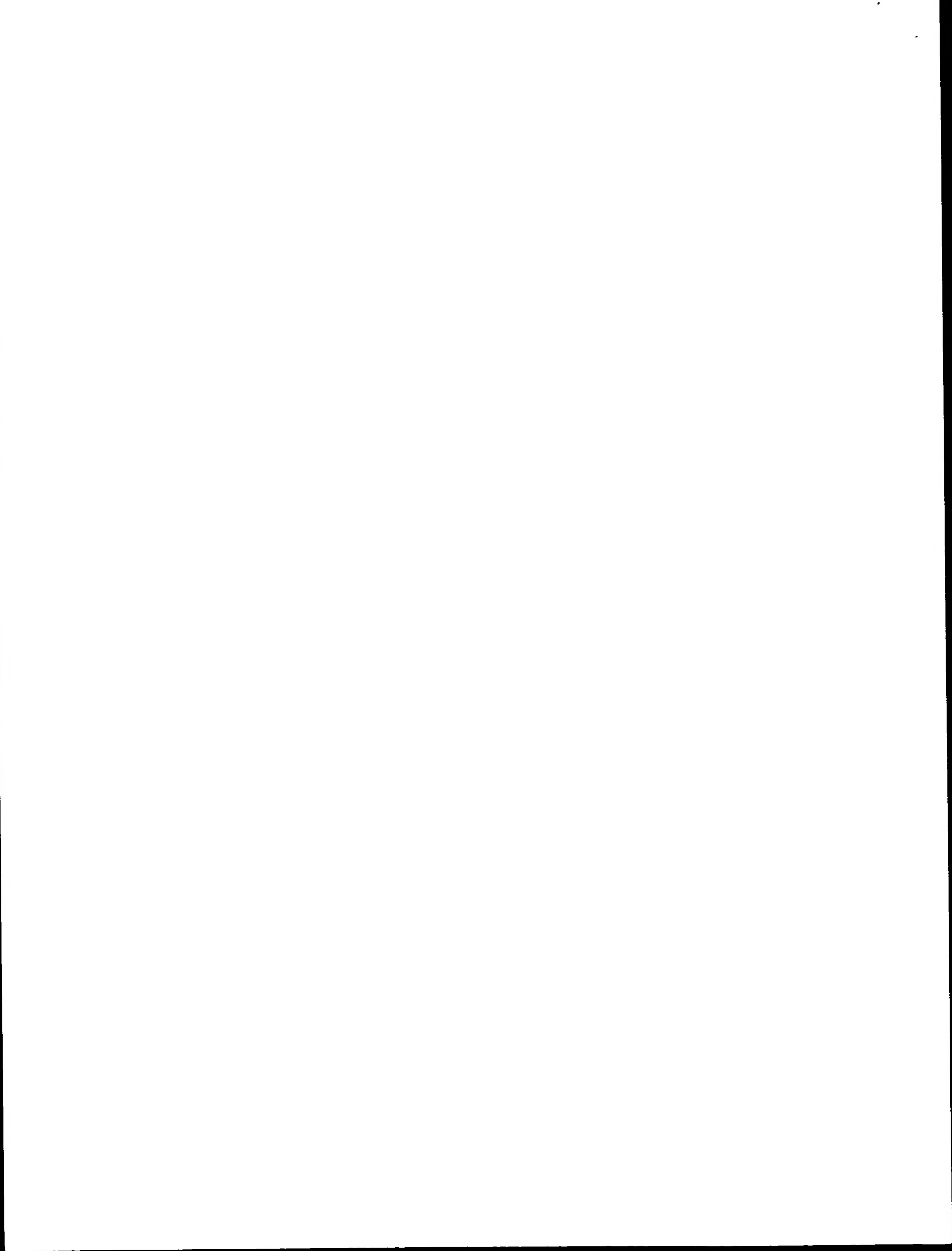
KEY	NAME	PARAMETER	GATE
■	091595.006	FL3-H	NO GATE POS
—	091595.005	FL3-H	NO GATE NEG



KEY	NAME	PARAMETER	GATE
■	091595.008	FL3-H	NO GATE POS
—	091595.007	FL3-H	NO GATE NEG

FIG. 18A

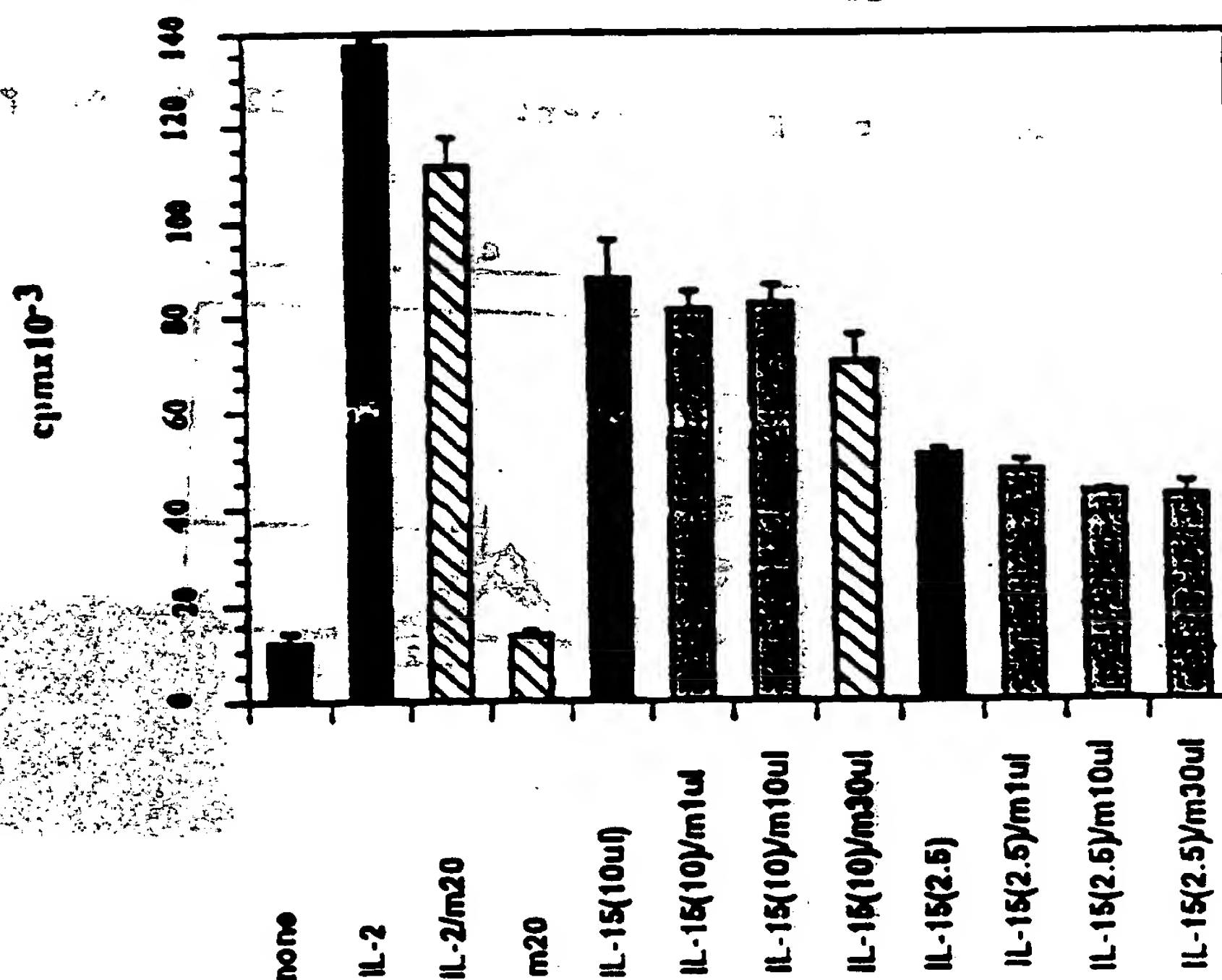
SUBSTITUTE SHEET (RULE 26)



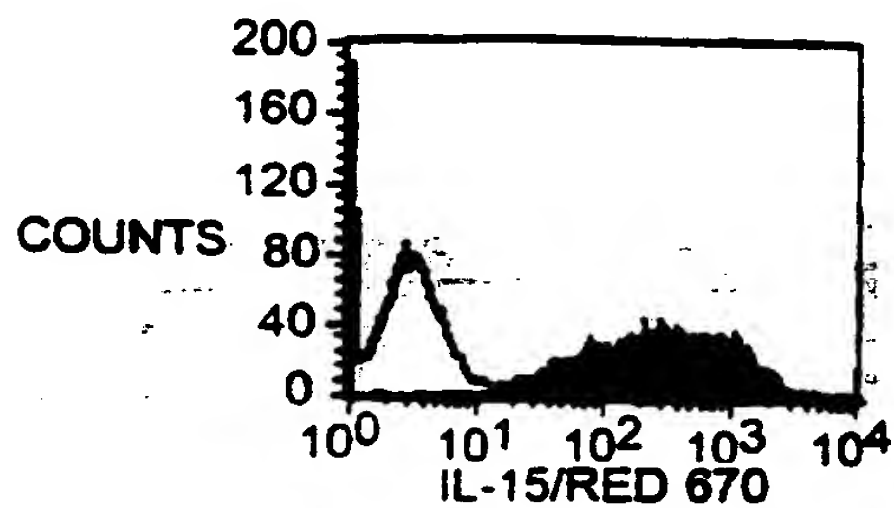
15/18

FIG. 16

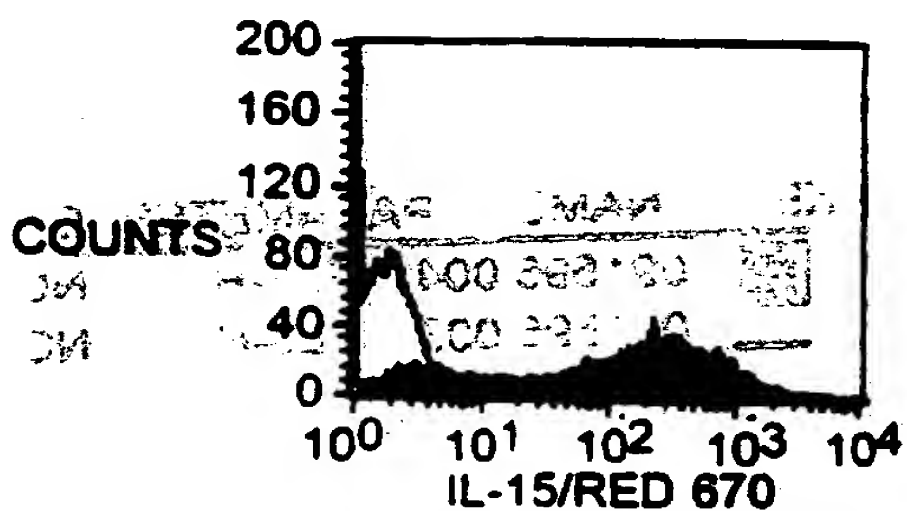
# The effect of mutant IL-15 on the proliferation of PHA stimulated human PBMCs



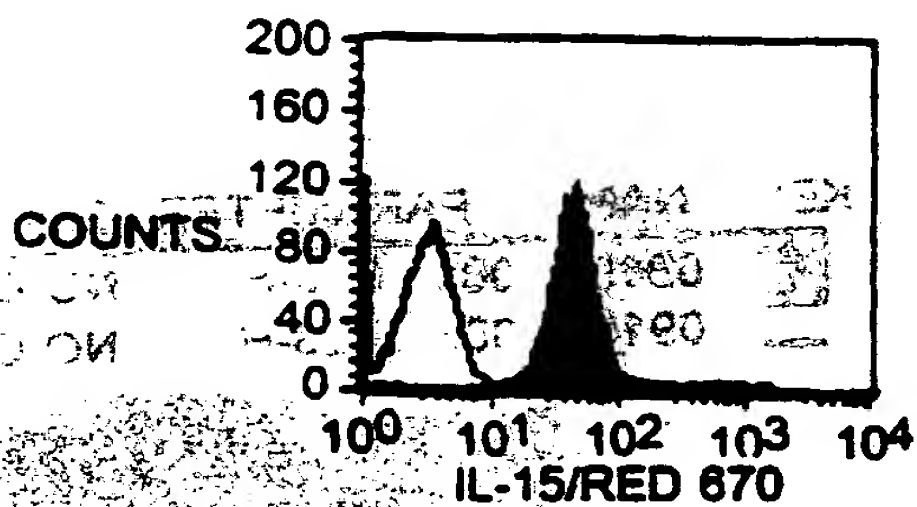
18/18



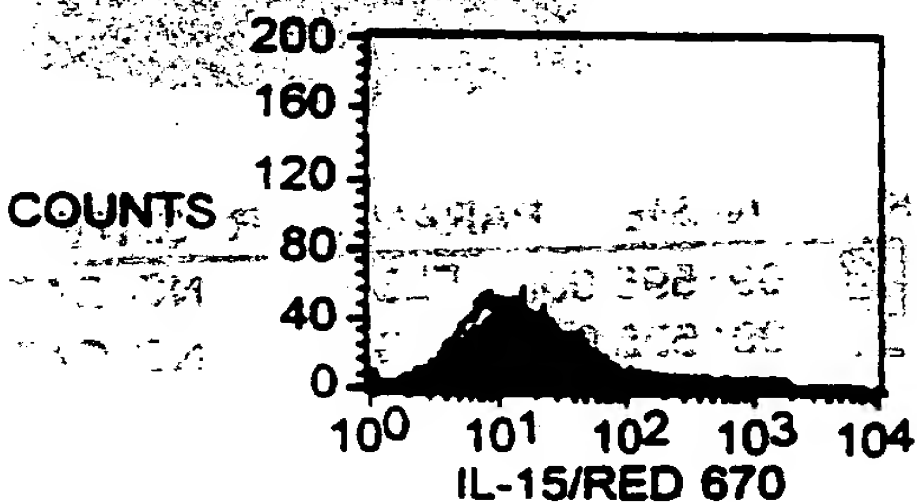
KEY	NAME	PARAMETER	GATE
■	091595.010	FL3-H	NO GATE POS
—	091595.009	FL3-H	NO GATE NEG



KEY	NAME	PARAMETER	GATE
■	091595.012	FL3-H	NO GATE POS
—	091595.011	FL3-H	NO GATE NEG



KEY	NAME	PARAMETER	GATE
■	091595.014	FL3-H	NO GATE POS
—	091595.013	FL3-H	NO GATE NEG



KEY	NAME	PARAMETER	GATE
■	091595.016	FL3-H	NO GATE POS
—	091595.015	FL3-H	NO GATE NEG

FIG. 18B

SUBSTITUTE SHEET (RULE 26)

# INTERNATIONAL SEARCH REPORT

Intern. Application No.  
PCT/US 97/06931

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/24 C07K14/54 A61K38/20 C12N15/62 G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 6 C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>PETTIT D K ET AL: "POLYETHYLENE GLYCOL CONJUGATION TO LYSINE RESIDUES OF RECOMBINANT IL-15 GENERATES A SPECIFIC IL-15 ANTAGONIST" PROCEEDINGS OF THE INTERNATIONAL SYMPOSIUM ON CONTROLLED RELEASE BIOACTIVE MATERIALS, vol. 22, page 496/497; XP000571219</p>	37
A	<p>GIRI J G ET AL: "UTILIZATION OF THE BETA AND GAMMA CHAINS OF THE IL-2 RECEPTOR BY THE NOVEL CYTOKINE IL-15" EMBO JOURNAL, vol. 13, no. 12, pages 2822-2830; XP000670218</p>	

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

### \* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- "Z" document member of the same patent family

Date of the actual completion of the international search

8 October 1997

Date of mailing of the international search report

15.10.97

Name and mailing address of the ISA

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Authorized officer

Le Cornec, N

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# INTERNATIONAL SEARCH REPORT

Intern Application No  
PCT/US 97/06931

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GIRI J G ET AL: "IL-15, A NOVEL T CELL GROWTH FACTOR THAT SHARES ACTIVITIES AND RECEPTOR COMPONENTS WITH IL-2" JOURNAL OF LEUKOCYTE BIOLOGY, vol. 57, no. 5, pages 763-766, XP000570466	
A	WO 96 04306 A (SCHERING CORPORATION) 15 February 1996 see claim 20 see page 21 - page 22	1-3
A	WO 95 27722 A (IMMUNEX CORPORATION) 19 October 1995 see page 8, line 21 - line 38; claims	1
A	M.A. BLANAR ET AL: "interaction cloning : Identification of a helix-loop-helix zipper protein that interacts with c-Fos" SCIENCE., vol. 256, 15 May 1992, DC3032 pages 1014-1018, XP002042889 cited in the application see the whole document	16,17
P,X	29TH ANNUAL MEETING OF THE AMERICAN SOCIETY OF NEPHROLOGY, 3 - 6 November 1996, NEW ORLEANS, LOUISIANA, USA, XP002042890 & D-W CHAE ET AL: "Mutant il-15 protein exerting antagonistic effects on il-15 triggered cell proliferation" JOURNAL OF THE AMERICAN SOCIETY OF NEPHROLOGY, vol. 7, no. 9, November 1996, page 1654 see abstract A2029	1-11, 16-21
P,X	WO 96 26274 A (IMMUNEX CORPORATION) 29 August 1996 see claims; examples 1,4 see page 2, line 21 - page 4, line 4	1-3,5-7
P,X	D.K. PETTIT ET AL: "Structure-function studies of Interleukin 15 using site-specific mutagenesis, polyethylene glycol conjugation, and homology modeling" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 272, no. 4, 24 January 1997, MD US, pages 2312-2318, XP002042891 see the whole document	1-3,5-7

# INTERNATIONAL SEARCH REPORT

International application No

PCT/US 97/06931

## Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 22-34 and 35  
because they relate to subject matter not required to be searched by this Authority, namely:  
remark: Although these claims (as far as in vivo methods are concerned) are directed to a method of treatment of the human/animal body (rule 39.1(IV) PCT), the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

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# INTERNATIONAL SEARCH REPORT

Information on patent family members

Intern. Application No

PCT/US 97/06931

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9604306 A	15-02-96	AU 3196195 A	04-03-96
WO 9527722 A	19-10-95	AU 680909 B	14-08-97
		AU 6767394 A	30-10-95
		EP 0772624 A	14-05-97
		FI 963828 A	02-12-96
		NO 964141 A	06-12-96
WO 9626274 A	29-08-96	AU 5027596 A	11-09-96